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The Tohoku Journal of Experimental Medicine, Vol. 51, Nos. 3 & 4, 1949.

Physiological Evidence for the Three Components-Theory of Color Vision.

By

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(Received for publication, November 7, 1947)

The theory of Young-Helmholtz is based on the facts of color mixtures. Young supposed that in the retina there are three types of nerve fibers which produce a sensation of red, green and blue respectively. The various color sensations result from the relative strengths with which the three kinds of fibers are stimulated, and a colorless sensation of white results if they are all excited equally. Expressed in modern terms, the three species of nerve fibers assumed by Young may be considered as three species of cones. Each type of cone contains its own particular photosensitive substance, and is concerned with an optic nerve fiber, stimulation of which produces the color sensation corresponding to it.

Helmholtz[1] assumed in each cone three different activities (chemical, electrical or other processes), because he became aware that the facts of visual acuity necessitated a smaller unit than three cones. In spite of the further elaboration of this theory since Helmholtz, there is no physiological evidence of the existence of three kinds of nerve fibers, cones or activities, except the experiments of color mixing. The data of color mixing themselves can be adequately described on the basis of tetrachromatic theories of Hering[2] and others as well, as pointed out by Schrödinger.[3] Furthermore, these theories are even more natural and simpler from a psychological point of view; they treat yellow and white as unitary sensations, because we can perceive no trace of red and green in yellow, and no trace of red, green and blue in white, although the trichromatic theory claims that yellow and white are compound sensations.

However, the unitary nature of white as a sensation does not hinder it from being the result of psychical fusion of two or more separate physiological processes. Therefore, it is neither psychological experiments nor measurements of color mixing that decide the question as to which of these views is correct. The problem is essentially a physiological one.

The author has shown in a series of papers[4][5][6] that the effect of

colors upon the retina shows itself in changes of the electrical excitability of the retina. In the present communication, this phenomenon has been made use of to prove that in reality, there are three kinds of physiological processes in the retina, as the three components-theory assumes.

Results.

1. Observations on Subjects of Anomalous Color Vision.

The general method is described in my preceding papers cited above, so that only points special for this experiment will be mentioned. As shown in my previous work,[4] the electrical excitability of the eye as measured by a rheobasic current becomes supernormal after an illumination. The maximum of excitability is reached usually in about two seconds after the end of illumination when white light is used.

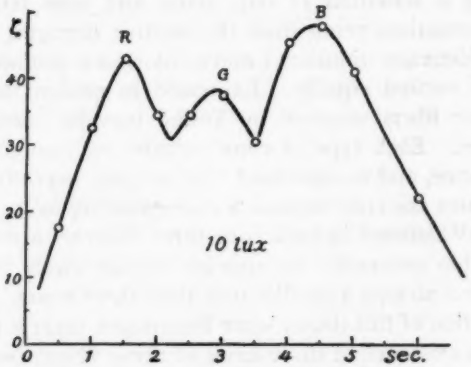

Fig. 1. Excitability curve of the eye of a deuteranomalous person (K. A.) for white light. Ordinates: Increases in electrical excitability (reciprocal of threshold) in percentage of the excitability in the dark without preceding illumination. Abscissæ: Time intervals between the end of illumination two seconds in duration and the stimulation with a constant current 100 msec. in duration.

It has been found that the time course of electrical excitability is quite different in persons of anomalous color vision from that in normal subjects. Particularly interesting for the present problem are the curves obtained from deuteranomalous persons, for they have three maxima, as if they corresponded to the three component-processes as hypothesized in the trichromatic theory. An example is illustrated in Fig. 1. The illumination was of an intensity 10 lux and confined to the center of the eye (the visual angle 2° in diameter). If the time interval between the end of illumination and the maximum of excitability is designated " crest time,"

each maximum has a definite crest time independent of intensities and wave-lengths of light (the crest time of a fused curve can vary with wave-length as previously reported). Although the crest time is so fixed the height of the maximum depends greatly upon intensities and wave-lengths.

The question now arises why the curve has three maxima in deuter-anomalous persons, while it has only one in normal trichromats. This question will be explained without difficulty, if it is assumed that there are three independent processes occurring in the retina, as is assumed in the three components-theory. The facts that each maximum has its own crest time, and that the relative heights of the three maxima depend on the wave-length of light accurately conform to the scheme of the theory.

In subjects of normal color vision, the relative magnitudes of the three component-curves must be such that they form a single congruent curve as a whole. Let the component curves of the shortest, intermediate and longest crest time be denoted by alphabets R, G and B respectively, then the curve G must be highest of all in order that three curves may fuse together into a curve with a single maximum. If the curve G happens to be reduced in its height, the three maxima must be brought to light as the result of incomplete fusion of the three component-curves. In deuter-anomalous subjects, the development of the G-process is not sufficient for the complete fusion of the three curves, so that the three maxima are clearly visible.

This interpretation is not only in good harmony with the statement of the three components-theory, but also justifiable by experimental inhibition of the G-process in normal subjects, as will be described later.

The results obtained at another subject K. S. of deuteranomalous vision are shown in Fig. 2. The measurements were made with three kinds of intensities, 180, 2.8 and 0.056 lux in order to see the effect of intensities on the time course of the excitability curve. This subject was well accustomed to this sort of experiments, and his data are more reliable than those shown in Fig. 1. The curve for white light of 180 lux shows distinct three maxima, the crest time of which are 0.75, 2.5 and 4 seconds. The values of crest times are a little different from those of the above subject, but these differences may be regarded as individual differences, so that it may be admitted to use the same notations R, G and B for the three maxima in this case. As seen from Fig. 2, the height of each maximum decreases as the intensity of illumination is lowered, but the crest times remain unaltered. It is especially worth noticing that the rate of decrease in heights is not the same for the three kinds of maxima; the curve G is most sensitive to decreases in intensity, what is evidenced by the fact that at the intensity 2.8 lux the maximum G is hardly visible while the other maxima are distinctly seen. Judging from the shape of the excitability curve it seems as

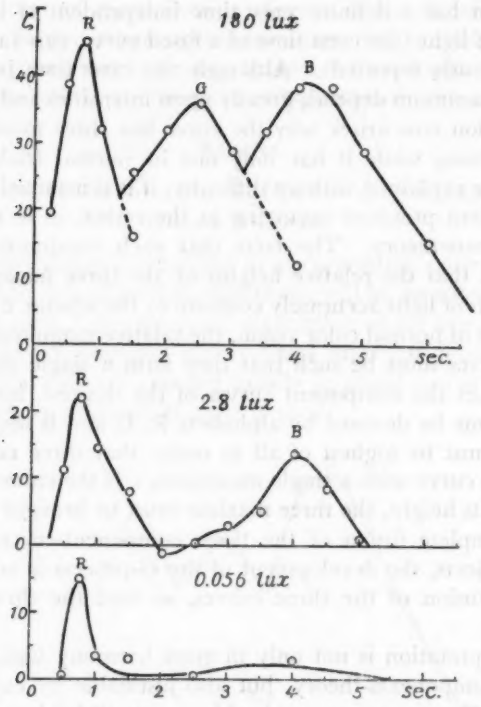

Fig. 2. The effect of intensities of white light upon the excitability curves at a deuteranomalous subject (K. S.) Ordinates: Excitability increases due to illumination with white light in percentage of the excitability without preceding illumination. Abscissæ: Intervals between the end of illumination and electrical stimulation.

if this subject were dichromatic in vision at this intensity. This fact corresponds to the results of psychological experiments that hue discrimination depends greatly upon the intensities of illumination, and that anomalous trichromats become practically dichromatic at low intensities.

Another fact worth while mentioning is that in this subject two different values of ordinates were often obtained for one and the same abscissa. One of the two values fell on one curve, while the other fell on the other kind of curve. This fact indicates that each curve behaves independently of the others, and that the fusion of the component curves found in normal subjects is only apparent one.

2. Demonstration of Three Kinds of Processes in the Retina of Normal Trichromats.

As seen from Fig. 3 (curves of empty circles and empty triangles), the

excitability curves for white light have only a single maximum, probably because the three processes R, G and B are excited in such proportions as the three curves apparently fuse together into a congruent curve. If such proportions are unbalanced in one way or another, the fusion will become less complete, and consequently the three maxima will be disclosed.

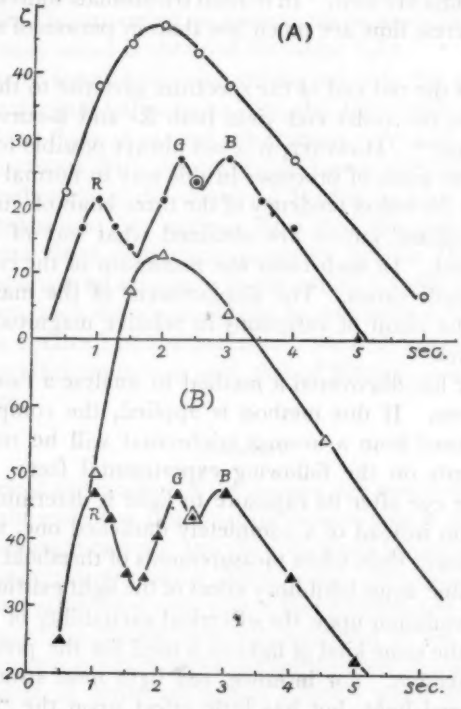

Fig. 3. Experiments showing the existence of three kinds of retinal processes in normal trichromats. (A): Excitability curve for white light (empty circles) and that for a monochromatic light 510 mμ in wave-length (solid circles). Subject: T. M. (B): Usual excitability curve for white light (empty triangles) and the curve for white light at one and the same subject under the influence of a weak green light (530 mμ) at the moment of electrical stimulation. The G-component is partially inhibited by the green light, and consequently the three maxima appear. Double circles and double triangles indicate the coincidence of two measurements.

According to the three components-theory, the relative strengths with which the three components are excited, must be different from wave-length to wave-length. Therefore, the three maxima must be revealed by illuminating with a monochromatic light of some adequate wave-length.

It was found that a bluish green light was adequate for this purpose, because it excites all three components in such a way that G- and B-component are stimulated to about the same degree. An example is shown in Fig. 3 (A). As seen from this figure, the B-curve is as high as the G-curve so that the two cannot fuse together. The crest times for R, G and B are 1, 2.25 and 3 seconds respectively. These values are always the same so long as the three maxima are seen. In normal trichromats individual differences concerning the crest time are much less than in persons of anomalous color vision.

Lights from the red end of the spectrum give rise to the R-curve only, while those from the violet end yield both R- and B-curves, as shown in the previous paper.[6] However, it is not always possible to prove the existence of the three kinds of processes in this way in normal trichromats, for in some of them the fusion tendency of the three kinds of curves is so strong that only monophasic curves are obtained what sort of monochromatic light may be used. In such cases the maximum of the curve is displaced as the wave-length varies. The displacement of the maximum is to be interpreted as the result of variations in relative magnitudes of the three component-curves.

The author has discovered a method to analyse a fused curve into its component-curves. If this method is applied, the compound nature of any curve obtained from a normal trichromat will be revealed at once. The method rests on the following experimental fact: When electrical threshold of the eye after its exposure to light is determined in an dimly illuminated room instead of a completely darkened one, the height of the curve is much lower than when measurements of threshold are made in the dark. This is due to an inhibitory effect of the light existing at the moment of electrical stimulation upon the electrical excitability of the retina. For the inhibition, the same kind of light as is used for the preceding illumination is most effective. For instance, red light most strongly reduces the value of ζ for red light, but has little effect upon the ζ-value for other kinds of lights. As has been stated above, the excitability curve for violet light has usually two maxima R and B, and it is the R-maximum that undergoes inhibition in the presence of red light at the moment of electrical stimulation. These facts suggest that the inhibitory interaction takes place only between the component-processes of one and the same kind.

By making use of this phenomenon it is possible to disclose the compound nature of the excitability curve for white light of any normal trichromat. If the threshold is determined under the illumination with a weak green light, the G-component will be inhibited most severly so that the three maxima must appear for the same reason as has been stated for the data of deuteranomalous persons. An example is shown in Fig 3 (B).

The curve shows distinct three maxima, the crest times of which are 1, 2.25 and 3 seconds. These values coincide with those of the previous case, suggesting that there is little individual difference among normal trichromats.

There is now no doubt that the appearance of three maxima in deuteranomalous persons is due to the congenital weakness of the G-component, for it has experimentally proved that three maxima are disclosed by partial inhibition of the G-component in normal trichromats in whom otherwise only a monophasic curve is obtained for white light.

In subjects of anomalous color vision, not only the proportions of the three processes are less harmonious, but also the values of crest times deviate greatly from those of normal trichromats. At any rate, our findings are in entire harmony with the theory of Young and Helmholtz, whereas they are contradictory to the theory of Hering at least in regard to the preripheral mechanism; our findings show that the process for white can be analysed into three component-processes against Hering's view that there should be a special substance in the retina for the reception of white light. However, such discussion should be restricted to the findings at the fovea, for data obtained at the periphery seem to agree neither with the theory of Young-Helmholtz nor with that of Hering, as will be reported in detail elsewhere.

Summary.

The electrical excitability of the human eye was measured at varying intervals after the end of an illumination two seconds in duration by means of a rheobasic constant current, and the differences between the excitabilities with and without the preceding illumination were plotted as ordinates against time intervals between the end of illumination and electrical stimulation as abscissæ. The illumination was restricted to the fovea centralis.

1. The curve so obtained has a single maximum in normal trichromats, but three maxima in deuteranomalous subjects when white light is used for illumination. The height of each maximum depends greatly upon intensities of illumination, but the crest time is entirely independent of intensities. The appearance of the three maxima is so interpreted that one of the three component-processes is too weak for such complete fusion as is found at normal subjects.

2. In some normal trichromats the three maxima appear in the excitability curve for a monochromatic light of some adequate wave-length. It was found that bluish green light is adequate for this purpose. Red light gives rise to a excitability curve with a single maximum, the crest time of which is about 1 second. The curve for blue light is also monophasic, the crest time being about 3 seconds. Violet light causes two

maxima, one of which has the same crest time as the curve for red light, and the other of which corresponds to the maximum for blue light.

3. When electrical threshold is determined in the presence of a weak green light after an illumination with white light, the three maxima appear in normal subjects like in deuteranomalous persons. This is due to an inhibitory effect of the green light upon one of the three component-processes.

All above findings provide physiological evidence of the three components-theory of Young-Helmholtz.

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(1) Helmholtz, H., Handbuch der physiologischen Optik (3 rd ed.)3 Vols,. Hamburg & Leipzig.

(2) Hering, E., Grundzüge der Lehre vom Lichtsinn. Berlin.

(3) Schrödinger, E., Sitzgsber. d. Wien. Akad. d. Wiss., Math.—Nat. Kl., 1925, **134**, ii a, 471.

(4) Motokawa, K., Tohoku J. Exp. Med., 1949, **51**, 165.

(5) Motokawa, K., Tohoku J. Exp. Med., 1949, **51**, 179.

(6) Motokawa, K., Tohoku J. Exp. Med., 1949, **51**, 197.

The Tohoku Journal of Experimental Medicine, Vol. 51, Nos. 3 & 4, 1949.

Biochemical Studies on Carbohydrates.

CXIV. Hexosamine Compounds in Lung.

Third Report. Heparin as a Component of Glycoproteins.

By

Motoyuki Utusi.

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Director: Prof. H. Masamune.)

(Received for publication, November 11, 1947)

Masamune and Yasuoka[1] put forward the opinion that carbohydrates, which are composed of hexosamine and hexuronic acid such as chondroitinsulfuric acid and mucoitin, take part in making up glycoprotiens in the sense of Masamune.[2] Since heparin belongs to the same group of carbohydrates, we undertook to examine if their assertion is applicable also to this compound. For this purpose a Fr. A obtained as in the first report,[3] involving heparin and the hemopoietic glycidamin, was dissolved in water and the portions of this solution precipitated at various pH's. In the precipitate at pH 2.4 was associated more of heparin with the glycidamin than in that at pH 4.0, resembling the relations in case of chondromucoid and funis mucin.[1] However, at pH 1.4, the glycidamin alone was thrown down and the centrifuged supernatant liquid gave intensive biuret test besides Neuberg and Saneyoshi's for glucuronic acid.[4] Probably the heparin-glycidamin salt formed at this pH is soluble as are the umbilical mucins at pH less than 1.8 and only the part in excess of the insoluble glycidamin came out.

Experimental.

Preps. I's. 200 mgms. of an A fraction were dissolved in 5 cc. of water with the aid of Na_2CO_3 (pH of the solution, 6–7) and 10% acetic acid was added to pH of 4.0, whereupon precipitation occurred. Centrifuged. The centrifugate was washed with a dilute acetic acid at similar pH, then with 95% alcohol and ether and dried constant in a vacuum desiccator over $CaCl_2$. Yields in two runs were 80 and 70 mgms.

Preps. II's and III's. They were prepared likely as above at pH's 2.4 and 1.4, by acidifying the solutions with drops of 2% HCl. 80 and 70 mgms. of the precipitates were obtained at the higher pH from two 200 mgms. portions of the material and at the lower pH 64 and 60

mgms. from 400 mgms. material each.

Analysis gave the figures in Table I.

TABLE I.

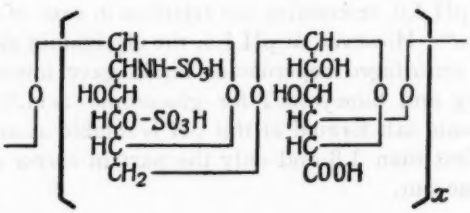
Composition of the Heparin-involving Glycoproteins.

Figures express the values in per cent.

pH of precipitation	Preparation \ Analysis	N*	Glucosamine†	Glucuronic acid	Galactose	Ash
4.0	I_a	12.18	16,02	6.10‡		2.98
	I_b	12.70	15.87	5.85‡		3.09
	Average	12.44	15.95	5.98		
2.4	II_a	11.18	18.00	8.38§		2.65
	II_b	11.45	17.43	8.40§		2.70
	Average	11.32	17.72	8.39		
1.4	III_a	13.45	12.29		13.06//	2.85
	III_b	13.64	12.68		13.69//	
	Average	13.55	12.49		13.38	

* Micro Kjeldahl. † Hamasato and Akakura method. ‡ Tanabe furfural method. § Osaki method[6]. // The average values by the indole, orcinol and diphenylamine I methods. In Preps. III's Neuberg and Saneyoshi test resulted in negative.

When we assume free heparin expressible in diagram like



namely its molecular formula as $(C_{12}H_{19}O_{16}NS_2)_n$[6], nitrogen, glucosamine and glucuronic acid in it are to amount to 2.82, 36.02 and 39.03% respectively. If then, the glucuronic acid content indicates that the precipitates at pH 4.0 are made up approximately of 15% heparin and 85% glycidamin and those at pH 2.4 of 21% heparin and 79% glycidamin. Therefrom in the former precipitates nitrogen is calculated at 12.0(=0.4+11.6)% and glucosamine at 16.2(=5.4+10.8)%, and in the latter nitrogen at 11.4(=0.6 +10.8)% and glucosamine at 17.6(=7.6+10.0)% (Regarding the nitrogen and glucosamine content of the glycidamin cf. the second report[7]), which agree fairly well with the findings. Preps. III_a and III_b gave no naphthoresorcinol reaction of hexuronic acid and their analytical values were similar

to the corresponding values of the pure hemopoietic glycidamin in the second report.

I desire to thank the Grant Committee for Scientific Researches of the Education Department for a grant towards the costs of this work.

Bibliography and Notes.

1) Masamune and Yasuoka, Tohoku J. Exp. Med., 1947, **49**, 181.

2) Masamune, ibid., 1949, **50**, 107.

3) Utusi, ibid., this volume, p. 101.

4) The centrifuged supernatant fluids at other pH's also showed the positive reaction in both of the tests.

5) Osaki, Tohoku J. Exp. Med., 1947, **49**, 15.

6) In a previous article (Masamune, Suzuki and Kondoh, J. Bioch., 1940, **31**, 343) we pronounced, based on the ash amount in the preparation, that one of the two sulfate rests in the heparin molecule takes the form of esteramide, but we overlooked at that time the possibility of the occurrence of an acid salt when heparin is prepared according to Chargaff and others. So we retract the claim until our study now in progress offers a final decision. H. M.

7) Utusi, Tohoku J. Exp. Med., this volume, p. 175.

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Thiamin Deficiency and Blood Sugar. Is there a Discrepancy between Human and Animal Findings?

Preliminary Report.

By

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(Received for publication, November 23, 1949)

It is considered as a well known fact that in case of thiamin deficiency hyperglycemia occurs. We have been investigating blood sugar content in many lactating womens. Though these were apparently healthy, there were 15 cases of normal Arakawa's reaction, about 60 cases of intermediate reaction and about 120 cases of almost completely or completely negative Arakawa's reaction. Now human milk negative to Arakawa's reaction is milk from a thiamin-deficient body.[1] Strongly or normally Arakawa-positive cases are to be considered as free of thiamin deficiency. If we presume, basing upon the well known fact about mentioned, blood sugar content in each group, the result would be like the following:—

Arakawa-positive group	Intermediate group	Arakawa-negative group
Normal sugar content	Slight hyperglycemia	Hyperglycemia

Our actual result[2] (mgrms. per 100 c.c.) was:—

Arakawa-positive group	Intermediate group	Arakawa-negative group
98.3	**93.5**	100.6

How shall we explain our result?

Now we have within our knowledge a couple of references (by Y. Okukawa and by J. Usuki) concerning blood sugar content in animals fed on thiamin deficient food. Their concurrent result is: In the initial stage of thiamin deficiency *hypoglycemia will occur*, and only in more advanced stage hyperglycemia will come about.

BLOOD SUGAR CONTENT

	Normal	Thiamin deficiency	
		Initial stage	Advanced stage
Human / Animal	Normal blood sugar	*Hypoglycemia*	Hyperglycemia

Thus it is clear that also in human cases *a hypoglycemic value* will be seen *in the initial stage of thiamin deficiency.*

And it will also be seen how sensitive Arakawa's reaction is by way of diagnosis for beginning thiamin deficiency.

Literature.

1) Cf. Many reports (published since 1930) on the Peroxidase Reaction, Tohoku J. Exp. Med. 2) N. Kaiho, 258th Report on the Peroxidase Reaction, to be published in a coming issue of this Journal.

The Tohoku Journal of Experimental Medicine, Vol. 51, Nos. 3 & 4, 1949.

Biochemical Studies on Carbohydrates.

CXV. Hexosamine Compounds in Lung.

Fourth Report: Glycidamins as a Component of Nucleoproteins.

By

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(From the Medico-Chemical Institute, Tohoku University, Sendai.
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(Received for publication, November 11, 1947)

Since Steudel it is generally believed that nucleoproteins are salts of a nucleic acid with a basic protein. But of those separated from various sources, many are obscure regarding the nature of the involved protein and do not contain nitrogen more than 16%. The examples are those from spleen, bone-marrow, hepatopancreas of octopus, submaxillary gland, placenta, brain and blood serum.[1] The proteins in these nucleoproteins are most probably the acidic, when analogized from the case of glycoproteins in the sense of Masamune.[2] We[3]-[6] have found that "glycoproteins" separate out, when tissue extracts are acidified, by combination of an acid protein with a hexuronic acid-containing polysaccharide through the ionized basic radicles of the former and the acidic of the latter. Acid proteins are suspected to participate also in making up nucleoproteins by a similar mechanism. Since, among acid tissue proteins, glycidamins are most readily extractible because of their having a carbohydrate in their molecule, these particularly might have opportunities to contribute to the production of nucleoproteins, and as a matter of fact, we were often perplexed to eliminate the accompanying nucleic acid, when isolation of pure glycidamins was aimed at as in studies of sclera mucoids[7], the mucin of stomach mucosa[8] and others.

The present experiment was so schemed in order to endorse the above supposition by taking advantage of the glacial acetic acid-soluble fraction (B fraction in the first report[9]) of a lung extract. Its solution in dilute methanol was precipitatdd by acidulation. In the precipitate were present both the nucleic acid and group specific glycidamin. On the other hand, the free nucleic acid was isolated by means of $Ba(OH)_2$ from the same material. Analysis of these substances showed that 3/4 part of the nucleoprotein here was occupied by the glycidamin.

Experimental.

The Nucleic Acid.

300 mgms. lots of a B fraction of lung were dissolved in 8 cc. of water[10] and drop by drop of the saturated $Ba(OH)_2$ was added. The precipitate occurring at neutral reaction was discarded and that at pH 9.4 was collected. The latter was suspended in 10 cc. of water and dissolved by acidifying with 3% HCl till pH 3.4, then the insoluble part centrifuged off and the supernatant liquid neutralized with 2% NaOH. Deposits appeared again. From the clear centrifuged supernatant fluid was the nucleic acid recovered by precipitation similar to above. It was washed with 95% alcohol and with ether and dried (in vacuo over $CaCl_2$). Crop, 76 mgms.

In this barium salt was shown the presence of phosphorus (ammonium molybdate test), sugar (Molisch reagents gave a bloodred colour tinged with violet), pentose (aniline acetate test) and purine bases (ammoniacal $AgNO_3$ test), but the substance did not give biuret and hexosamine (Osaki and Turumi procedure) reactions. Quantitatively, it contained 13.60% N (micro Kjeldahl), 6.68% P (Plimmer[11]) and 14.60% ash. In order to measure the optical rotatory power, the substance was dissolved in water by the use of hydrochloric acid. The pH of the solution was 5.6. $[\alpha]_D^4 = \frac{1.10 \times 3.0125}{0.0181 \times 1.0024 \times 1} = +182.6°$.

When it is assumed that the phosphate rests of the present nucleic acid possess each only one non-chemically-bound OH as in thymo- or yeast-nucleic acid and are combined fully with Ba in the salt, the composition of the acid in free state is calculated at: N, 15.91%; P, 7.82%.

The Nucleoprotein.

600 mgms. from the material used above were purified by redissolution in glacial acetic acid (extracted with a 5 cc. and a 3 cc. portion of the solvent) and reprecipitation of the clear centrifuged supernatant fluid with acetone. After washing with acetone and ether and drying, the amount recovered was 400 mgms. This was taken up in 10 cc. of water[12] and 5 cc. of 97% methanol (More of methanol caused precipitation) and subsequently 30% acetic acid were added. The precipitate, which came out at pH 4.5, was centrifuged, washed with acid dilute methanol (similar to the mother liquid as to dilution and pH), then with 95% alcohol and ether and dried.

Two specimens were thus prepared. About 250 mgms. were yielded in each case.

Tests. Biuret, Molisch, Osaki and Turumi (by both the direct and indirect processes), ammonium molybdate (for phosphorus), ammoniacal

$AgNO_3$ and aniline acetate tests resulted in positive.

Analysis gave results in Table I.

TABLE I.

Composition of the Nucleoprotein.

Figures express the percentages.

Analysis	Sp. I	Spe. II	Average
N	13.18	13.25	13.22
Glucosamine	10.55	10.38	10.47
P	2.10	1.93	2.02

Computed separately from the content of phosphorus and of glucosamine (the group-specific glycidamin contains 13.99% glucosamine[13]), it is given that 1/4 (26%) of this nucleoprotein is taken up by the nucleic acid and 3/4 (75%) by the glycidamin. Nitrogen of the nucleoprotein is calculated from this proportion at 13.5% (nitrogen of the glycidamin, 12.72%), coinciding nearly with the values found.

We are indebted to the Grant Committee for Scientific Researches of the Education Department for a grant to us. H. M.

Bibliography and Notes.

1) Readers are referred to Steudel (Hoppe-Seyler and Thierfelder, Handb. d. physiol. u. path.-chem. Analyse, 9th Ed., Julius Springer, Berlin, 1924, 533–541).
2) Masamune, Tohoku J. Exp. Med., 1949, **50**, 107.
3) Hasegawa, ibid., 1943, **46**, 95.
4) Oh-Uti, T., ibid., 1948, **49**, 193 and 207.
5) Asagi, ibid., 1949, **50**, 95.
6) Masamune and Yasuoka, ibid., 1947, **49**, 181.
7) Osaki, ibid., 1947, **49**, 19.
8) Ozaki, J. Bioch., 1937, **26**, 239; Sibuya, Tohoku J. Exp. Med., 1949, **50**, 355.
9) Utusi, Tohoku J. Exp. Med., this volume, p. 101.
10) A clear solution was obtained.
11) Cf. Haneda, Sinokawa and Yasuoka, Tohoku J. Exp. Med., 1944, **46**, 263.
12) The substance dissolved perfectly.
13) See the second report (Tohoku J. Exp. Med., this volume, p. 175).

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Is there Any Case with Yakriton-deficiency or Deficiency of the Detoxicating Liver Hormone? What is the Use of Yakriton in Clinical Medicine?

Preliminary Report.

By

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(Received for publication, November 23, 1949)

What is the purpose of treatment with the detoxicating hormone[1] of the liver? Is there any case of yakriton-deficiency? If so, how is the case diagnosed?

The usual (subcutaneous) dose of yakriton is 1/3～2 R.A.U. (Rabbit-Ammonia-Units). But it is highly probable that within the body we have[2] in store much more than such an amount of the hormone. However, yakriton in a preformed state in the body is comparable to a river streaming on a plain, which cannot be utilized for exciting electricity for practical purpose. For this purpose we must give the river a necessary amount of descent. The preformed yakriton in the body is comparable to the river on a plain, while injection of yakriton is something like giving that descent.

Insulin, for instance, is usually used in case of diabetes—in case of insulin insufficiency; in this case the hypoglycemic convulsions are not desirable. But the acute hypoglycemia curve is the purpose of insulin shock therapy for those not suffering from any insulin insufficiency.

The preexisting yakriton in the body does only the daily routine work of just disposing* with toxic metabolites occurring physiologically. But it cannot work effectively enough in time of emergency. Injected yakriton will develop its excellently detoxicating effect according to its *different amount*—number of R.A.U. (Rabbit-Ammonia-Units). It is almost incredible that a rabbit, probably with more than 10 R.A.U. of yakriton within its body, should succumb to acute phosphorus poisoning, when the animal can be *saved from it with a prophylactic injection of* 1/4 *R.A.U.* of yakriton.

Literature.

1) Cf. Many reports (published since 1926) on Yakriton, Tohoku J. Exp. Med.
2) A. Sato, Tohoku J. Exp. Med., 1949, **50**, 206.

* This is not the place for discussing the function of the preexisting yakriton; this will be related in a coming issue of this Journal.

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A New Compound Excreted in Urine and Developing Colour Reaction with *p*-Dimethylaminobenzaldehyde.

First Report.

By

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(Received for publication, December 18, 1947)

In urine are already known various compounds which react with Ehrlich's *p*-dimethylaminobenzaldehyde reagent. Urobilinogen, Freudenberg's anti-sheep hemolysin (a glucosamine-containing polysaccharide), Jorpes' anti-isoagglutinin (a glycidamin) and urinomucoid (Kobayasi) give the reaction immediately without any preliminary treatment and kynurenine after oxidation with H_2O_2. During the course of our investigations on those group-specific substances, we became aware of the presence of another compound, which is similar in behaviour toward the reagent, in the filtrate of the basic-lead precipitate of the anti-sheep hemolysin or of corresponding carbohydrates of B and O urine. Speparation of it to establish its nature has been tried over and over again, all but with failure and regretfully no decisive claim can be put forward at present. However, its several properties examined will be worthy of description for a further investigation:—

1) It was ultrafiltrable (collodion membrane) as well as dialyzable (animal membrane).

2) Soluble not only in water but also in absolute alcohol and butanol[1] and partially extractible into acetone and pyridine from its solid mixtures with other urine components. Insoluble in acetic ester and ether.

3) As dissolved in water it was scarcely adsorbed to activated charcoal and kaolin.

4) When heated with hydrochloric acid in advance, it formed on treatment with alkaline acetylacetone solution a structure, which gives a red colour with the Ehrlich reagent. The spectrum of this colour[3] exhibited an absorption band from λ 5700 to 5200 Å that divided in a short while (15′) into two bands, one lying at 5700 Å and the other ranging from 5300 to 5200 Å.

It also gave a light red coloration by the indirect process of Osaki and Turumi[3] but not by the direct.

5) It was readily oxidized by mercuric oxide at neutral and alkaline reactions in the cold and rendered incapable of showing the colour reaction in 4).

6) Its output was larger when the urine was more acid. 9 normal individuals excreted 29 (pH of the urine, 6.8)—334 mgm. (pH of the urine, 5.6) in a day (Table III). In normal rabbit urine, which is alkaline, it appeared scarcely, but in urine at pH of some 6 of those rabbits to which menthol or β-naphthol had been injected noticeable amounts of it were found (Table II).

An example of fractionation trial of urine to separate the substance will be given in the experimental part.

Experimental.

Determination Procedure.

The procedures of Yositake[4] and of Hamasato and Akakura[5] for hexosamine determination were combined as follows.

First method.

20 cc. of urine (acidulated with acetic acid when the urine is alkaline) are precipitated with 4 cc. of 37.9% lead acetate solution. The filtrate is removed from lead with the aid of H_2S and after aeration off of H_2S, ultrafiltered through a collodion membrane (Zsigmondy apparatus). The ultrafiltrate is freed from acetic acid by passing ether in a continuous extractor for 1.5 hours and 6 cc of it heated together with 4 cc. of 2 n. HCl in a 25 cc.-containing ampoule (hard glass) at 150°C (in an oil bath) for 75 minutes. Cooled. The digestate is transferred perfectly into a beaker with the aid of two 5 cc. portions of 25.7% NaCl solution. And 250 mgm. of stannous chloride are dissolved and subsequently sodium carbonate powder added until the solution becomes to pH of 5.6. The solution is made up to 25 cc. in a measuring flask with water and filtered and this filtrate subjected to colorimetric analysis according to Hamasato and Akakura, after addition, when necessary, of a smallest requisite quantity of glucosamine hydrochloride to cause sufficient colour development.

By 75 minutes digestion mentioned of the ultrafiltrate is the maximum value obtained. In urine of an individual, for instance, the colour appeared in depths corresponding to 0.050, 0.083 and 0.05 mgm. of glucosamine, when 1 cc. of the digestates after heating for 60, 75 and 90 minutes was adapted to colorimetric analysis (Colorimetry was carried out after addition of 0.5 mgm. glucosamine hydrochloride per cc. of the digestates and the amount of glucosamine added was subtracted from the values

found). This duration of heating appears to sufficiently decompose urea and other urine components, which interferes with the colour reaction, and longer heating to gradually break up the substance in question, when considered from the results obtained on ovomucoid added to urine (Table I). Ovomucoid was chosen here for reasoning by analogy because the unknown substance may be of complex nature giving the colour reaction only after hydrolysis as are mucoids. That this mucoid is hydrolyzed completely

TABLE I.

Recovery of Glucosamine Added in the Form of Ovomucoid.

300 mgm. of ovomucoid (glucosamine content, 14.5%) were added to 20 cc. of human urine. Glucosamine in 1 cc. of the heat digestate, that was subjected to colorimetric analysis, amounted to 0.52 mgm.

Time of heating min.	Colour development as hexosamine by 1 cc. of the digestate		
	Found mgm.	Recovery of ovomucoid-glucosamine	
		mgm.	In per cent.
30	0.490		
60	0.555	0.505	97
75	0.600	0.517	99
90	0.556	0.506	97
120	0.400		

in 75 minutes was proved by heating its watery solution with hydrochloric acid under similar conditions. The urine used was the one noted on above.

Second method.

In later determinations the precipitation of urine with lead acetate and subsequent ultrafiltration were substituted with single operation of treatment with lead subacetate, namely:—

10 cc. of urine is alkalized with 5% ammonia water to pH 9.0–10.0 and followingly just an enough quantity of the saturated lead subacetate added to throw down all the precipitable substances. The precipitates are filtered off on a Büchner funnel and washed with two 2 cc. portions of water. The filtrate and washings are combined and bubbled with H_2S gas and lead sulfide separated on a filter with suction and washed with two 2.5 cc. portions of water. The filtrate and washings here are acidified together to pH 1–2 with drops of 10% H_2SO_4 and ether passed through. They are then neutralized with 10% NaOH (a few drops) and evaporated under reduced pressure to dryness. The remainder is dissolved in 10 cc. of water and subjected to heating with HCl and following operations described in the former method.

Trials to Increase the Excretion of the Substance in Rabbit Urine.

To start with, menthol, β-naphthol and phenol were given to rabbits. Menthol was administered (2/3–1 gm. per kg. bodyweight) per os as dissolved in lukewarm water by means of a Nalaton's catheter, and phenol (0.25 gm. per kg. bodyweight) as 1% solution in cold water. β-Naphthol was given by forced ingestion (1 gm. of its powder per kg. bodyweight) with a small amount of crushed carrot.

The analysis was made on the whole-day urine. When menthol was given, the pH of the urine was lowered to round 6.0 in about 1 week after the beginning, and the output of the substance increased simultaneously. This raised excretion continued as long as the urine remained at or below this pH, even after the administration was stopped. An example is illustrated in Table II.

TABLE II.

Excretion of the Substance in Question in the Whole-day Urine, When Menthol was Administered per os to a Grown-up Rabbit (♀).

Date	Bodyweight kg.	Menthol administered gm.	Volume of urine cc.	pH of urine	Output as hexosamine mgm.
Jan. 15	3.09	2	835	8.0	almost nil
16	2.87	"	690	7.8	
17	3.11	"	775	7.8	
18	3.01	"	575	6.5	9.8
19	2.86	3	728	6.5	
20	2.87	"	585	6.4	9.0
21	3.04	"	590	6.4	112.1
22	3.02	"	600	6.2	
23	3.05	"	400	6.2	152.0
24	3.05	"	515	6.2	
25	2.86	"	125	6.0	68.8
26	2.70	"	60	5.4	60.0
27	2.70	0	50	5.2	
28	2.70	0	50	5.2	180.0
29	2.70	0	50	5.2	

During a long application of β-naphthol only temporary (a few days) increase of the output took place with acidification of urine. Phenol was ineffective at all in both of the respects.

In normal human urine, which is acid, were found comparatively large quantities of the substance varying in parallel with the strength of acidity, as is demonstrated in Table III.

From these findings it might be plausibly assumed that the excretion of the substance is promoted by acidosis, and so, to afford a further evidence, subcutaneous injection of neutral inorganic salts ($CaCl_2$, NH_4Cl, NaCl, Na_2SO_4 and Na_2HPO_4) and peroral application of HCl (0.1 and 0.2%) were continued day after day on rabbits, but the urine

did not change acid.

TABLE III.

Excretion of the Substance in Question in the Whole-day Human Urine.

Name, sex and age (year)	Body-weight kg.	Volume of urine cc.	pH of urine	Amount of the substanse as expressed as hexosamine (mgm.)	
				In 1 cc of urine	In whole-day urine
K.H., ♂, 39	54.0	1,450	5.6	0.23	333.5
K.T., ♂, 34	49.5	1,670	6.0	0.08	136.6
E.I., ♂, 40	54.0	1,700	6.0	0.10	170.0
S.T., ♂, 23	60.6	1,300	6.2	0.069	89.7
M.U., ♂, 31	54.2	1,620	6.2	0.058	94.0
Z.Y., ♂, 27	60.4	1,800	6.4	0.029	52.2
K.O., ♂, 34	52.5	1,600	6.4	0.029	46.4
Y.H., ♂, 33	51.2	1,700	6.4	0.029	49.3
N.H., ♂, 33	49.1	1,630	6.8	0.017	29.4

Fractionation of Urine.

A trial of isolating the substance, though fruitless, will be given for example. 1 *l.* of acid rabbit urine (pH, 5.2), involving 900 mgm. as hexosamine of the unidentified compound, was collected from four rabbits to which menthol had been given. The urine was alkalized with ammonia water and the saturated basic lead acetate solution added as in analysis (Second method). The filtrate from the precipitates was removed from lead with H_2S. After eliminating acetic acid with ether, it (pH, 5.6) was condensed under reduced pressure to a syrup and extracted with absolute alcohol repeatedly by boiling under reflux. Into the extracts, amounting to 150 cc. in total and condensed to 100 cc., 70 cc. of 20% alcoholic solution of oxalic acid were poured to precipitate urea, and the centrifuged supernatant liquid boiled under a return condensor with $BaCO_3$ powder, added in portions, until pH of the solution was elevated to 4.8. When cold, barium oxalate and barium carbonate were separated off on a funnel and the filtrate decolorized with activated charcoal (1 spoonful) (This solution will be referred to as A. E.) and evaporated at a low temperature to a syrup. The residue was dissolved with two 50 cc. portions of water. Analysis showed that the substance had been reduced to 495 mgm. The solution reacted neither with naphthoresorcinol (test for hexuronic acid) nor with aniline acetate (test for pentose) but gave the positive Molisch test. Colorimetry with orcinol and indole and measurement of iodine reduction gave figures which coincided with one another as calculated as glucose.

The watery solution was again evaporated and the remaining syrup boiled with three 50 cc. portions of acetone (boiled each time for 1 hour), then the extracts united (323 mgm. of the substance came over) and after distilling off the solvent, the rest was taken up with changes

of cold water (60 cc. altogether). The combined solution was concentrated to 10 cc. and analyzed. 290 mgm. of the substance were found in it. The colour developed thereby showed a spectrum having a band, which divided later into two bands already noted. When the determination procedure was followed by replacing Reagent A of Hamasato and Akakura with this reagent less acetylacetone (the salt solution), the colour reaction did not happen. The residue unextracted by acetone above gave the positive reaction of the indirect Osaki-Turumi test. In it hydrolysable sulfur and sugar (Molisch test) were detected. But Ehrlich diazo reaction of urochromogen and Ehrlich-Bauer aldehyde reaction of urobilinogen resulted in negative, although tested immediately as well as after heating with HCl.[6]

The whole remaining (8 cc.) of the watery solution of the acetone-soluble fraction was condensed to a syrup and fractioned with pyridine (Two 10 cc. portions of this solvent were used and boiled each time for 30 minutes), but again the substance did not accumulate in one fraction (about half of it dissolved).

Crystalline products were obtained during condensation of the extracts at various stages above, but all of them gave no colour reaction by the determination procedure.

An attempt of isolating the substance as an acetyl derivative was made by taking an alcohol-soluble fraction of another urine corresponding to A. E. in the above but was also useless.

Exclusion of the Possibility of the Substance to be Kynurenine.

10 cc. of an A. E., which had been prepared as described and contained 40 mgm. as hexosamine of the compound searched for, were evaporated, the residue taken up in 2.5 cc. of water and kynurenine was precipitated with mercuric sulfate and washed with 6% mercuric sulfate solution according to Kotake and Kawase.[7] The precipitate was then suspended in 10 cc. of water, kynurenine liberated by means of H_2S and H_2S aerated off. The solution obtained gave no colour with the Ehrlich reagent both by the Osaki and Turumi indirect process and the analytical procedure (the heating with HCl and condensation with acetylacetone included), while it gave the positive reaction of Otani and Honda.[8] On the other hand, the supernatant liquid after centrifugation of the kynurenine precipitate was removed from mercury in a like way and from H_2SO_4 with $Ba(OH)_2$. This liquid gave the colour reaction by the Osaki and Turumi indirect process and analysis indicated it contained 35 mgm. of the unknown substance as hexosamine.

Thanks are due to the Grant Committee for Scientific Researches of the Education Department for a grant in aid to us. H. Masamune.

Bibliography and Notes.

1) Extracted from water in Dakin's apparatus.
2) The spectrum of the corresponding dye of glucosamine shows a band from λ 5340 to 4750Å.
3) Osaki and Turumi, Tohoku J. Exp. Med., 1947, **49**, 11.
4) Yositake, J. Bioch., 1939, **30**, 199.
5) Hamasato and Akakura, J. Bioch., 1941, **34**, 159.
6) The digestate smelled of menthol distinctly.
7) Kotake and Kawase, Zeits. physiol. Chem., 1933, **214**, 6.
8) Otani and Honda, Osaka Igakkaishi, 1938, **37**, 1.

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Studies on the Antibiotic Substances from Actinomyces.

(3rd Report)

On the Identification of the Antibiotics from Actinomyces by the Specificity of Culture Filtrates (so-called "secondary screening" of 1st group of actinomyces).

By

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83 representative strains out of 201 proved to be effective upon gram-positive, -negative and acif-fast bacteria by streak plate method ("primary screening" test) were cultivated on the surface of Waksman's media and the culture filtrates were tested on their activities and other biological properties, by broth dilution method, cup assay method and one-dimensional diffusion method using 12 kinds of test bacteria, including E. coli, streptomycin resistant E. coli, Sta. aureus (Terashima and 209 strains) and others ("secondary screening" test).

By the different inhibitory ratios against Sta. aureus and E. coli, by the different activities in acidic and alkaline test media, by the different antibacterial spectra, and by the different diffusibilities, active culture filtrates obtained from 83 strains were subdivided into several types, i.e. streptomycin, streptothricin 1, streptothricin 2, streptothricin 3, acidic and neutral type.

Several kinds of known and unknown antibiotics were isolated from the culture filtrates of the representative strains of each type and purified into crystalline forms. On the chemical and biological significances of each antibiotic the reports will be made by each investigators elsewhere.

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Studies on Desiccation of B. C. G. Vaccine, especially regarding the Medium and Vacuum Grade.

By

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Introduction.

The B.C.G. vaccine, used for the prevention against pulmonary tuberculosis at present, is an emulsion of living bacilli, and its useful period is very short. Moreover, it must be kept in an equipment of low temperature for the purpose of preservation and transportation. In order to overcome such inconvenience, therefore, I have made a trial manufacture of a small-sized apparatus for the desiccation and attempted the drying of B.C.G. vaccine in vacuo from the frozen state.

The method of making dried B.C.G. vaccine by means of a small-sized apparatus.

The small-sized apparatus, designed by myself, is for the most part made of hard glass. The structure made some improvements on Flosdorf-Mudd's[1] drier consists of a manifold, to which two condensers are attached, measuring equipments of vacuum grade (Geissler's tube and Mc Leod's vacuum gauge), a mercury diffusion pump, a container of absorbents, and a Cenco Hyvac pump. And at the same time, we can join ten ampoules to the small-sized manifold. Proceeding with the study of dried B.C.G. vaccine, which was manufactured by means of this apparatus, I must explain briefly the process of its manufacture.

First, prepare a homogeneous emulsion of B.C.G., transplanted on Sauton's liquid from glycerol bile potato and then promoted the growth for ten to fourteen days at the third generation of the culture on the liquid media, with a medium by hand grinding method or by the action of ultra sound waves (conditions of the action: grid current 24–32 mA, anode voltage 2200–2800 volt, high frequency current 700–900 mA,

anode current 220–260 mA, tuning regulator 3.0–3.8, heating regulator 5–6, output regulator 3–4, action time 15 minutes and the density of this vaccine is at the rate of 10 mg or 5 mg of B.C.G. in 1 cc. of medium.) and pour 1 cc. of the vaccine into an ampoule of ordinary glass of 50 cc. or 10 cc., and then freeze it about 15 minutes in a freezing mixture (about −20°C) of calcium chloride and scrap ice. Next, quickly attach that ampoule to a coupler tube of above mentioned manifold, previously evacuated and then kept the vacuum grade of 10 mm^{-2} of mercury, and open the cock.

The vaccine dries up in about 5 to 8 hours without melting because of self-freezing, at under 15°C of temperature in our drying-room. The water of the vaccine is caught by calcium chloride in the container of absorbents. When the temperature of our drying-room was over 16°C, I kept the temperature of outside of the ampoule at about 10°C by the use of a chest of continual low temperature with the mixture of salt and scrap ice, so as to prevent the melting of the freezing vaccine. For the purpose of preservation of such dried B.C.G. vaccine, I sealed up the ampoule in vacuo (every time generally 10^{-2} mm of mercury) at the neck, and, in case it is necessary to seal in high vacuum, in combination with a mercury diffusion pump. Also, to confirm the maintenance of vacuum in the ampoule, I performed the unpolar discharge by means of an equipment for the examination of vacuum grade.

The examination method of the number of living bacilli in dried B.C.G. vaccine.

1) Re-emulsify the dried B.C.G. vaccine in an ampoule in the density just before drying with physiological saline solution (ph=6.2–6.4).

2) Dilute further the re-emulsified vaccine from 1 mg to 10^{-6} mg (bacterial dose of B.C.G. that 1 cc. of each dilute vaccine contains) by means of the dilution method of decuple progress. In this case, I changed each pipette for dilution with another sterilized one at every progress.

3) Next, pour slowly 0.1 cc. of such dilute vaccine into the Oka-Katakura's[2] glycerol egg slants in test tubes respectively from density to density, stuff their mouths with cotton and lay them on their sides in an incubator for a night, then seal them with cork and wax instead of the cotton.

4) Next calculate the number of colonies that grow on the slants just after a month of culture in an incubator, and denote it by signs of ⋕ (beyond count) or ⋕ (over 50).

On the medium for the making of dried B.C.G. vaccine.

In reference to the medium, Shitama[3] reported that 1% dextrin and

Kirchner's solution were comparatively good, but they were still opened to discussion for putting them to practical use. Hayashi[4] also made a memoir that media of saccharoid could generally keep the living B.C.G. long, and 5% milk sugar was likely to be the most hopeful in the respect that it gave a comparatively good homogeneity to its re-emulsified vaccine.

By way of my preliminary examination, I made many sorts of B.C.G. vaccine with seventeen media (solutions of 3–5% milk sugar, 1% pepton, 1% asparagin, 1% dextrin, 3–5% glucose, 3–5% bouillon, Kirchner, physiological saline, 0.3% gelatine, 5% galactose, 0.1% agar, 0.1% starch, 5% cane sugar, distilled water, Lockemann's solution, 5% serum, and Sauton's solution) by the hand grinding method that gives comparatively little injury to living B.C.G. and examined the state of re-emulsifying and the existence of living bacilli just after drying. Then I selected eight media (solutions of 1% asparagin, 3% milk sugar, 1% pepton, 0.5% bouillon, 3% glucose, 1% dextrin, Kirchner, and physiological saline) of them that showed relative good results and made each dried B.C.G. vaccine with them (ph always 6.8) by the action of ultra sound waves. Then I performed regular examinations still more to investigate the quality.

As the results of experiments are given in Table I, the dry time was the shortest in 1% pepton or physiological saline solution, and the longest in 1% dextrin. The external appearance of dried B.C.G. vaccine is very different. The medium of dried B.C.G. vaccine, the surface of which had the appearance of reticulation, sponge, and pumice stone, and which did not adhere to the wall of an ampoule, was, in general, likely to be better than the others.

As for the investigation of the re-emulsifying state, I poured physiological saline solution into the ampoule that contained dried B.C.G. vaccine to observe its soluble velocity, then examined its homogeneity with the naked eye and through a microscope, after shaking the emulsion for about ten minutes. The results were good in the order of 3% milk sugar, 1% pepton, 1% dextrin, and 1% asparagin.

Next the number of living B.C.G. in each vaccine of 1% asparagin, 3% milk sugar, and 1% pepton, was almost the same degree as just before and after drying. Therefore I preserved them in an incubator at 37°C from four to eight weeks and tested them on the number of living B.C.G. and the re-emulsifying state, and, as shown in Table II, I knew that 1% pepton was the best.

TABLE I. Dried B.C.G. vaccines with various kinds of media (1st).

No	Medium	Time	External appearance	Re-emulsifying state		Examination of the number of living B.C.G.							
				Soluble Velocity	Homogeneity	Comparison	Bacterial dose and Colony numbers						
							10^{-1} mg	10^{-2} mg	10^{-3} mg	10^{-4} mg	10^{-5} mg	10^{-6} mg	10^{-7} mg
I	1% Asparagin	6.5 hours	reticular	Considerably rapid	good	Contrast	||| ||| ||| ||| ||| 19	2
						Dried	||| ||| ||| ||| ||| 20	1
II	3% Milk sugar	7 hours	minute bubble	immediately	good	Contrast	||| ||| ||| ||| ||| 20	1
						Dried	||| ||| ||| ||| ||| 12	1
III	1% Pepton	6 hours	fine reticular	almost without delay	good	Contrast	||| ||| ||| ||| ||| 28	0
						Dried	||| ||| ||| ||| ||| 18	0
IV	0.5% Bouillon	6.5 hours	reticular	late	somewhat good	Contrast	||| ||| ||| ||| ||| 19	2
						Dried	||| ||| ||| ||| 23	3	0
V	3% Glucose	7 hours	bubble (adhered to the ampoule)	Considerably rapid	bad	Contrast	||| ||| ||| ||| ||| 5	0
						Dried	||| ||| ||| ||| 18	7	1
VI	1% Dextrin	8 hours	like a fine pumice stone	almost without delay	good	Contrast	||| ||| ||| ||| ||| 23	2
						Dried	||| ||| ||| ||| 13	1	0
VII	Kirchner	6.5 hours	somewhat like wheat-gluten (adhered to the ampoule)	Considerably late	somewhat good	Contrast	||| ||| ||| ||| ||| 12	2
						Dried	||| ||| ||| ||| 23	1	0
VIII	Physiological saline solution	6 hours	stratified (adhered to the ampoule)	very late	very bad	Contrast	||| ||| ||| ||| 30	3	1
						Dried	||| ||| ||| 22	3	0	0

* Notes: Time: the time required for vacuum desiccation. The temperature of the drying-room is 10°C~15°C. External appearance: the appearance of dried B.C.G. vaccine. Comparison: the comparison between contrast (just before drying) and dried vaccine (just after drying). |||: beyond count, ||: over 50.

TABLE II.

Notes: Time: the time required for vacuum desiccation. The temperature of the drying-room is 10°C~15°C. External appearance: the appearance of dried B.C.G. vaccine. Comparison: the comparison between contrast (just before drying) and dried vaccine (just after drying). ##: beyond count, #: over 50.

TABLE II.

Dried B.C.G. vaccines with various kinds of media (2nd).

Kinds of Vaccine \ Medium \ Bacterial dose	1% asparagin (colony number)							3% milk sugar (colony number)							1% pepton (colony number)						
	10^{-1} mg	10^{-2} mg	10^{-3} mg	10^{-4} mg	10^{-5} mg	10^{-6} mg	10^{-7} mg	10^{-1} mg	10^{-2} mg	10^{-3} mg	10^{-4} mg	10^{-5} mg	10^{-6} mg	10^{-7} mg	10^{-1} mg	10^{-2} mg	10^{-3} mg	10^{-4} mg	10^{-5} mg	10^{-6} mg	10^{-7} mg
undried (contrast)	##	##	##	40	5	0	0	##	##	##	##	##	20	0	##	##	##	##	40	6	1
dried (just after drying)	##	##	##	5	1	0	0	##	##	##	##	##	12	0	##	##	##	##	10	0	0
,, (preserved for 4 weeks)	##	##	5	1	0	0	0	#	5	1	0	0	0	0	##	##	33	4	1	0	0
,, (preserved for 8 weeks)	30	2	0	0	0	0	0	0	0	0	0	0	0	0	##	##	#	2	0	0	0

Notes: Dried vaccines were preserved in an incubator at 37°C.

The influence of conditions of sealing the ampoule on dried B.C.G. vaccine.

It has been recognized since olden times, by Rogers[5] and many other authors, that it is necessary to seal up the vessel of dried bacteria in vacuo, in order to preserve them alive for a long time. In connection with dried B.C.G., Ogasawara[6] sealed the ampoule at about 10 micron of vacuum grade at the end of desiccation; Shitama[2] and Hayashi[4] sealed that respectively at about 5×10^{-3} mm and 3×10^{-2}–3×10^{-1} mm of mercury.

For the purpose of studying in detail that subject, I examined how dried B.C.G. vaccine is influenced by such conditions of sealing as mentioned below. That is to say, having sealed ampoules of B.C.G. vaccine at the necks in high vacuum (5×10^{-6}mm–$\frac{5}{2}\times$ 10^{-3} mm of mercury), or in vacuum (10 mm-^{2}–3×10^{-2} mm of mercury), or after the substitution of air at the end of desiccation, and then preserved them in an incubator at 37°C from four to eight weeks, I experimented on the re-emulsifying state and the number of living B.C.G. in them.

As the results of the experiment are shown in Table III, the dried B.C.G. vaccines

TABLE III.

The influence of the sealing conditions of ampoules on dried B.C.G. vaccine (1st).

Medium	Vaccine number	The sealing conditions of ampoules	The temperature and duration of preservation	Bacterial dose and colony numbe						
				10^{-1} mg	10^{-2} mg	10^{-3} mg	10^{-4} mg	10^{-5} mg	10^{-6} mg	10^{-7} mg
1% pepton	I	unsealed	just after making	||| ||| ||| ||| 40	6	1
	II	,,	just after drying	||| ||| ||| ||| 10	0	0
	III	in high vacuum ($5\times^{-6}$ mm of mercury)	at 37°C for 4weeks	||| ||| ||| 14	1	0	0
	IV	in vacuum (10^{-2} mm of mercury)	,,	||| ||| 33	4	1	0	0
	V	in the substitution of air	,,	0	0	0	0	0	0	0
	VI	in high vacuum ($5\times^{-6}$ mm of mercury)	at 37°C for 8 weeks	||| ||| ||| 20	0	0	0
	VII	in vacuum (10^{-2} mm of mercury)	,,	||| ||| ||| 2	0	0	0
	VIII	in the substitution of air	,,	0	0	0	0	0	0	0
1% asparagin	I	unsealed	just after making	||| ||| ||| 40	5	0	0
	II	,,	just after drying	||| ||| ||| 5	1	0	0
	III	in high vacuum (5×10^{-6} mm of mercury)	at 37°C for 4 weeks	||| ||| 3	1	0	0	0
	IV	in vacuum (3×10^{-2} mm of mercury)	,,	||| ||| 5	1	0	0	0
	V	in the substitution of air	,,	0	0	0	0	0	0	0
	VI	in high vacuum (5×10^{-6} mm of mercury)	at 37°C for 8 weeks	19	0	1	0	0	0	0
	VII	in vacuum (3×10^{-2} mm of mercury)	,,	30	2	0	0	0	0	0
	VIII	in the substitution of air	,,	0	0	0	0	0	0	0
1% dextrin	I	unsealed	just after making	||| ||| ||| 20	5	1	0
	II	,,	just after drying	||| ||| 20	15	1	0	0
	III	in high vacuum ($\frac{4}{3}\times10^{-3}$mm of mercury)	at 37°C for 4 weeks	||| ||| 4	1	0	0	0
	IV	in vacuum (10^{-2} mm of mercury)	,,	||| ||| 12	1	0	0	0
	V	in the substitution of air	,,	0	0	0	0	0	0	0
	VI	in high vacuum ($\frac{4}{3}\times10^{3}$mm of mercury)	at 37°C for 8 weeks	||| 17	0	0	0	0	0
	VII	in vacuum (10^{-2}mm of mercury)	,,	1	2	0	0	0	0	0
	VIII	in the substitution of air	,,	0	0	0	0	0	0	0

Notes: 1 vaccine is an undried (contrast).

of ampoules sealed in high vacuum had generally a good number of living bacilli, but it did not differ much in comparison with those sealed in vacuum, and those sealed after the substitution of air showed no growth of colonies on the glycerol egg slants. With regard to the re-emulsifying state, the vaccines of ampoules sealed in high vacuum were generally better than the others, but most of them sealed after the substitution of air were bad, perhaps affected by oxygen and humidity. I also prepared four ampoules that contained dried B.C.G. vaccines, and sealed them in vacuo (about 10^{-2} mm of mercury).

Then I welded them quickly to an equipment for the substitution of nitrogen gas and evacuated them, and sealed the half of them in high vacuum again and the others in substituting the dried nitrogen gas, collected as purely as possible, under one air pressure. And all of them I preserved for seventy-six days or one hundred and thirty days in a dark room to make a comparative study. According to the results published in Table IV, the vaccines of ampoules sealed in high vacuum were some-

TABLE IV.

The influence of the sealing conditions of ampoules on dried B.C.G. vaccine (2nd).

Medium	Vaccine number	The sealing conditions of ampoules	The place and duration of preservation	Bacterial dose and colony number						
				10^{-1} mg	10^{-2} mg	10^{-3} mg	10^{-4} mg	10^{-5} mg	10^{-6} mg	10^{-7} mg
1% prepton	I	unsealed (contrast)	just after making	##	##	##	22	3	1	0
	II	,,	just after drying	##	##	##	6	1	0	0
	III	in high vacuum	in a dark room for 76 days	##	##	0	0	0	0	0
	IV	in the substitution of nitrogen gas	,,	40	0	1	0	0	0	0
	V	in high vacuum	in a dark room for 130 days	##	##	2	0	0	0	0
	VI	in the substitution of nitrogen gas	,,	35	4	0	0	0	0	0

what better than those in substituting nitrogen gas, with respect to the number of living B.C.G. Next, in order to investigate the difference between the vaccine of an ampoule sealed in high vacuum (5×10^{-6} mm of mercury or so) and that of an ampoule sealed in vacuum (10^{-2} mm of mercury or so) in case of preserving at room temperature which comes into the greatest question for the practical use of dried B.C.G. vaccine, I let them alone in the light place of our laboratory (about 7°C–35°C) for one hundred and seventy-five days, and then performed the comparative experiment. As Table V gives their results, the vaccine in vacuum was somewhat inferior to that in high vacuum, with respect to the number of

living B.C.G. and the re-emulsifying state, but made no great difference.

TABLE V.

The influence of the sealing conditions of ampoules on dried B.C.G. vaccine (3rd).

Medium	Vaccine number	The sealing conditions of ampoules	The place and duration of preservation	Bacterial dose and colony number						
				10^{-1} mg	10^{-2} mg	10^{-3} mg	10^{-4} mg	10^{-5} mg	10^{-6} mg	10^{-7} mg
1% prepton	I	unsealed (contrast)	just after making	###	###	###	24	1	0	0
	II	„	just after drying	###	###	###	33	6	1	0
	III	in high vacuum (5×10^{-6}mm of mercury)	in a light place of our laboratory for 175 days	###	###	21	2	0	0	0
	IV	in vacuum (10^{-2}mm of mercury)	„	###	##	6	0	0	0	0

Notes: The temperature of our laboratory was at about 7°C~35°C.

SUMMARY AND CONCLUSION.

1) The solution of pepton (1–2%) is the best medium for the making of dried B.C.G. vaccine.

2) The dried B.C.G. vaccines of ampoules sealed in high vacuum or in vacuum are both good for the purpose of preservation, and there is no great difference between themselves, but those of ampoules sealed in substituting nitrogen gas for vacuum are somewhat inferior to those in vacuum, and those of ampoules sealed in substituting air for it are the worst.

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Studies on the Water Metabolism in Diabetes Mellitus.

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Polyuria is a chief complication of diabetes mellitus, and it is well known that from the excessive loss of water there results a severe degree of desiccation of the skin.

Jaksch[1] who in 1893 first reported a failure of water metabolism in diabetes, pointed out the loss of water and the increase of desiccated substance of the blood; afterwards Klein[2] and Hoff[3] also noticed the similar result.

On the other hand, there were many reseachers[3-10] who reported the occurence of edema in diabetes mellitus. Moreover, we find many reports of the relationship between insulin and water metabolism; and it is generally accepted there is a definite water retention or restricted diuresis with the injection of insulin and so called "insulin edema."[2][11][12][13][14]

Further Meyer-Bisch[15] and others have reported of the water and mineral metabolism.

The following two methods have been generally accepted for the observation of the water metabolism in diabetes.

One is the observation of the blood concentration by means of hemoglobin determination and so on.[2][11][13][16][17][18]

The other is the measurement of output and specific gravity of urine after the administration of water. For example, Klein[16] and Gerbi[19] pointed out by Volhard's water test that the urine output is less in diabetic cases before treatment than in normal persons. Ralli and others[20] mentioned that in 8 cases of diabetic coma the amount of urine was very small in comparison with that of water taken.

In the present report we want to observe the water metabolism in diabetic patients, etiology of polyuria, the desiccation of the skin and edema.

Materials and Method.

Classification of diabetics in our Clinic is as follows: Type I which is

recovered with "high carbohydrate, low fat diet," Type II with application of insulin.

Cases which are brought to our attention are 5 cases of Type I, 14 cases of Type II and 5 normal persons as the control.

Usually the experiments begin early in the morning prior to breakfast. During the experiments no foods are given.

"Volhard's dilution test" is performed for the first 4 hours by intake of 1000 c.c. of water from the mouth and without interruption we enter the "concentration test" for the last 4 hours by giving 200 grms. of bread. After the administration, urine was taken ½, 1, 1½, 2, 3 and 4 hours in the former test, ½, 1, 2 and 4 hours in the latter, and its quantity, specific gravity, urine sugar and in some cases blood sugar were examined.

They were weighed prior to and 4 hours after the water intake.

The blood sugar was determined by the Fujita-Iwatake's method, the urine sugar by the Bertrand.

Results of Experiment.

We well summarized the results of experiment performed in normal individuals and diabetic patients in Table I, most of the latter were experimented before, but partially soon after, dietary management.

From the results of the control experiment performed in 5 healthy persons, we observed that the total amount of urine during the first 4 hours was 985 to 1223 c.c., and during the last 4 hours was 120 to 490 c.c.

For example, in Case 1, after taking water an increased urine output reached its maximum and specific gravity its minimum in 1½ to 2 hours, followed by a gradual recovery. After taking bread there was no marked increase of the urine output, however the specific gravity reached its maximum in ½ to 1 hour and no remarkable drop was detected.

2 cases gained, 2 cases lost in weight and 1 case remained unchanged.

We found no marked difference between the results of control experiment and those of Type I, showing that the amount of urine during the first 4 hours was 960 to 1490 c.c. and during the last was 133 to 505 c.c. During the former no urine sugar was found except in Case 8, although during the latter it was found in all cases.

Weight: 3 patients lost, 2 patients remained constant and no patient gained in weight.

In other all cases of Type II with the exception of 2 cases (Cases 11 and 12) in which we found more output than in normal persons and the loss of weight, it was interesting to note that after drinking water the amount of urine during 4 hours was very small, ranging from 120 to 780 c.c. and after eating bread it increased ranging from 285 to 810 c.c., and that the specific gravity which is generally high in this type showed no appre-

TABLE I.

Type	No. of case	Urine amount (c.c.) During the first 4 hours	Urine amount (c.c.) During the last 4 hours	Urine sugar (grms.) During the first 4 hours	Urine sugar (grms.) During the last 4 hours	Clinical examination several days before experiment: Blood sugar (mgrms. %)	Urine sugar (grms.)	Urine amount (c.c.)	Urine specific gravity
Control	1	1223	410	(−)	(−)	——	(−)	——	——
	2	1200	——	(−)	(−)	——	(−)	——	——
	3	1170	320	(−)	(−)	——	(−)	——	——
	4	1120	490	(−)	(−)	——	(−)	——	——
	5	985	120	(−)	(−)	124	(−)	1100—1700	1020—1030
I	6	1490	445	——	——	202	21—58	2000	1030
	7	1422	133	0	0.76	144	1—7	350—1700	1020—1030
	8	1125	190	3.82	11.49	284	29—81	1300—2500	1020—1030
	9	960	505	0	9.00	238	19—98	1400—2800	1015—1020
	10	960	260	0	2.68	180	0—19	2200—2700	1005—1018
II	11	2095	705	——	——	280	84—146	1750—1900	1030—1040
	12	1505	1115	26.23	38.16	286	75—209	1500—2500	1025—1030
	13	780	435	4.77	31.00	150	129—219	3100	1035
	14	690	460	0.26	19.35	188	80—159	1400—2000	1030—1040
	15	686	543	6.84	39.70	310	149—289	2200—4500	1033—1040
	16	535	285	1.00	19.27	316	93—135	1400—2050	1030—1035
	17	530	370	3.87	27.63	294	138—255	2600—4400	1030—1040
	18	508	360	1.10	22.85	252	69—163	1800—2570	1035—1040
	19	450	330	0.34	24.58	236	126—173	2000—3800	1030—1040
	20	330	810	8.80	68.76	297	168—485	3500—6000	1030—1040
	21	310	730	23.10	64.68	298	369—859	6700—12500	1040—1045
	22	275	365	1.50	26.33	346	97—206	2000—2700	1030—1040
	23	250	320	0.01	23.01	264	75—107	1400—1800	1030—1040
	24	120	433	6.49	38.46	303	334—440	4400—5800	1040—1043

ciable fall after the administration of water and no remarkable rise after the intake of bread.

It is a matter of course that the urine sugar was found in all cases during both the first and the last 4 hours.

Weight: 8 patients gained, 3 patients lost and 1 patient remained constant in weight.

After the treatment:

In about one half of these cases the same experiments were repeated in order to observe the influence of diabetic treatment on the water metabolism.

Results were indicated in Table II.

In Case 10 which received dietary treatment for 41 days, the urine output increased from 960 to 1275 c.c. during the first 4 hours and decreased in some degree during the last 4 hours.

In Case 13 which showed no remarkable effect in spite of dietary control for 13 days, a decreased urine output was observed during both the first and the last 4 hours, and the latter is especially striking.

In Case 14 which showed not only no disappearance of urine sugar but the rise of blood sugar with the use of insulin for 66 days, the urine output decreased during the first 4 hours and increased during the last 4 hours. On the contrary, in Cases 15, 22 and 24, in which we found a remarkable decrease of blood sugar and no urine sugar with insulin injection, there was a marked increase of urine output during the first 4 hours and decrease during the last 4 hours.

In Case 18, we found the same result as in previous 3 cases, with the exception of a slight increase during the latter.

In Case 20, we noticed no distinct contrast between the result of experiment before and after dietary treatment, and the same experiment without water which was repeated two days later indicated the same result as before contrary to our expectation.

From this fact, it is probable, that the administration of water has no influence upon the urine output.

While, in this case, after recovery with insulin treatment for 46 days with a marked decline of blood and urine sugar, we found an interesting fact that the amount of urine showed a conspicuous increase during the first and decrease during the last 4 hours.

In Case 21, the same result as in Case 20 was derived.

Blood sugar curve:

In 9 cases the blood and urine sugar were observed simultaneously; the gradual fall after the administration of water and rise after that of bread were found, and the severer diabetes were, the more remarkable the results were.

TABLE II.

Type	No. of case	Remarks	Urine amount (c.c.)		Urine sugar (grms.)		Clinical examination			
			During the		During the		several days before experiment			
		After giving	first 4 hours	last 4 hours	first 4 hours	last 4 hours	Blood sugar (mgrms. %)	Urine sugar (grms.)	Urine amount (c.c.)	Urine specific gravity
I	10	S.d. for 8 days	960	260	0	2.68	180	0—19	2200—2700	1005—1018
		S.d. for 41 days	1275	210	0	0	172	(—)	1900—3100	1015—1020
II	13	Full diet	780	435	4.77	31.00	150	129—219	3100	1035
		S.d. for 13 days	690	255	0.07	14.27	174	106—152	2000—2400	1035—1040
	14	S.d. for 7 days	690	460	0.26	19.35	188	80—159	1400—2000	1030—1040
		Ins. for 66 days	470	745	(+)	(+)	282	26—61	1100—1500	1020—1030
	15	S.d. for 7 days	680	543	6.84	39.70	310	149—289	2200—4500	1033—1040
		Ins. for 33 days	1358	74	0	0	124	(—)	1900—2400	1015—1025
	18	S.d. for 19 days	508	360	1.10	22.85	252	69—163	1800—2570	1035—1040
		Ins. for 13 days	858	420	3.41	9.35	184	0—30	1300—2200	1020—1030
	20	Full diet	330	810	8.80	68.76	297	168—485	3500—6000	1030—1040
		S.d. for 5 days	143	680	4.58	55.16	„	„	2000—3200	1015—1040
		S.d. for 7 days (without giving water)	215	600	6.93	55.08	„	„	„	„
		Ins. for 46 days	1040	148	0	0.50	111	0—3	1000—3300	1015—1020
	21	Full diet	310	730	23.10	64.68	298	369—859	6700-12500	1040—1045
		S.d. for 11 days	180	605	3.57	47.59	208	210—400	3150—5800	„
		S.d. for 10 days (without giving water)	153	500	1.14	20.24	„	„	„	„
		Ins. for 28 days	1475	225	0	4.24	111	0—8	1000—2000	1010—1020
	22	Full diet	275	365	1.50	26.33	346	97—206	2000—2700	1030—1040
		Ins. for 12 days	1013	219	0	9.32	234	0—44	740—1300	
	24	Full diet	120	433	6.49	38.46	303	334—440	4400—5800	1040—1043
		S.d. for 14 days	205	562	13.09	44.05	„	157—254	2100—3000	1043—1047
		Ins. for 12 days	1764	296	0	0	„	(—)	600—1300	1015—1030

S.d.: standard diet. Ins.: insulin.

SUMMARY.

Dilution test:

No remarkable difference between normal individuals and the patients of Type I was observed. The amount of urine output was approximately 1000 c.c., the specific gravity gradually decreased and the body weight was invariable or slightly lost.

On the contrary, in Type II there were no striking increase of output and fall of specific gravity except 2 cases.

The results of the above experiment correspond to that of Klein,[16] Gerbi[19] and Ralli.[20]

Moreover, it is interesting that the same results were obtained in the experiment performed without giving water; and it is assumed that in severe cases of diabetes the water intake has no influence upon the urine output and the fluid taken is stored in the body.

After recovery from diabetic symptoms by the suitable treatment, the urine output increased in every case, especially remarkable in severe cases notwithstanding the total daily amount becoming small, although we found no great difference in comparison with the results before the treatment when there was no recovery.

Concentration test:

In every case the rise of specific gravity was observed, while in Type II it is so high previously as a rule that it was not so marked.

The amount of urine is comparatively small in Type I.

As Kailhack[21] described, we found out a close relation between the amount of urine output and urine sugar, that is, when the former was large the latter was simillarly large and we noticed the decrease of the former together with that of the latter after recovery with the diabetic treatment.

Discussion:

As many authors stated, no established theory on the disturbance of water metabolism has been found.

They thought to find out the causal meaning in the disturbances of kidney function caused by glycosuria,[22] in diabetic acidosis,[3][6] in the failure of the intermediary water metabolism in the tissues,[15] in saline action,[23] in the insufficiency of nutrition of capillary wall due to the disturbance of carbohydrate assimilating process[7] and in hypophysis.[24]

In severe stage of diabetes, there occurs the profound dehydration from the excessive loss of water, and it is easily assumed that in the dilution test done under this condition the administrated water is stored in the body, and that by the administration of bread this stored fluid is excreted in a large quantity of urine which goes in parallel with the rise of blood sugar and the increase of urine sugar.

After all, severe diabetic patients have a tendency to store the fluid in the body cells preparing for excretion of an increased urine sugar following hyperglycemia.

If they take the food which causes hyperglycemia without taking the water sufficiently, the occurence of dehydration, clinically thirst and desiccation of the skin are observed.

On the contrary if they take the water sufficiently and the food restrictedly, the fluid is stored and clinically edema occurs.

In severe diabetic cases, as Nakagawa[9] reported, it is easily understood that we see frequently the occurence of edema in such cases as the restricted diet is given; while Stolte[8] said diabetic edema is due to cachexia caused by the excessive limitted diet, therefore, the food must be given sufficiently; although this is different from our opinion theoretically, we can easily endorse it.

Further, it is understood from these facts that it is seldom to see diabetic edema under the dietary management of our Clinic, containing 250–350 grms. of carbohydrate, 80–100 grms. of protein, 30–40 grms. of fat and about 1600–2200 calories.

Conclusions.

1. Concerning the dilution test which is done with normal persons and diabetic patients of Type I, there is no striking difference between them; on the contrary, in Type II, the amount of urine is very small.

2. After recovery from diabetic symptoms, it increases in every case, especially remarkable in severe cases in spite of the disappearance of polyuria.

3. In the concentration test, we find a parallel relation between the amount of urine and urine sugar.

4. In severe cases there is a tendency to store the water in the body preparing for excretion of an increased urine sugar following hyperglycemia. It is probable that when they take such food as causes hyperglycemia without taking the water sufficiently, clinically a severe degree of thirst and desiccation of the skin occur, on the contrary, when they take the water sufficiently and the food restrictedly, the fluid is stored in the body cells and edema occurs.

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Liver Function in Diabetes Mellitus.

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There is an intimate relation between the liver and pancreas embryologically, anatomically and from the point of internal secretion and digestive function.

The occurence of a secondary disturbance of the pancreas in liver and duct diseases was reported by some authors,[1][2] and I[3] had mentioned before there were many investigators in foreign countries who emphasized that gall stone may be one of the most important etiologic factors in diabetes.

On the contrary, the liver disorders due to the pancreas disturbance were noticed.[4]-[17]

On the other hand, some investigators observed such liver disturbances at necropsy in diabetes as changes of the liver weight[18] and glycogen content,[18]-[20] fat infiltration of the liver cells[18][21] and hypertrophy of the reticular fibers.[18]

However, I could not find so many reports on the hepatic function in diabetes, especially there are only a few reports concerning this problem in Japan.

The liver function was observed in 55 cases of diabetes using 6 tests; and in the present paper I want to report these results, especially an interesting fact obtained in Sodium Santonate test and to present the influence of diabetic treatment upon liver function.

Method of Investigation.

Tests applied for liver function are as follows: Sodium Santonate test (S test) and Azorubin-S test (A-S test) which I[22] described before, Hippuric Acid test after Ishiyama[23] in which the reading below 46.4% in the male and 44.1% in the female were accepted as pathological from my result of the control experiment, Millon's reaction, Takata's reaction and Urobilinogenuria.

Results of Experiment.

1) S test.

Concerning the detoxicating function of the liver in diabetes, there is only one report described by Koga[24] who found the liver injury in 45.5% containing suspect of 22 cases of pancreatic diabetes.

(i) Results.

Among 54 cases I found an increased output (the amount of Santogenin output) in 4 cases, a rise of excretory ratio (the amount of Santogenin output for 8 hours versus that for 4 hours) in 4 cases, a decreased output and rise of ratio in 2 cases, an increased output and a fall of ratio in 15 cases, an increased and decreased output in addition to delay of the first appearance in the urine in 1 case and 2 cases respectively, a delayed appearance, decreased output and a rise of ratio in 2 cases, an increased output, delayed appearance and a fall of ratio in 6 cases showing a suspect increase except 1 case, a delayed appearance and a fall of ratio in 1 case, an increased output, delayed appearance and a rise of ratio in 1 case.

As hepatic suspect disturbance 7 cases were indicated, among which 6 cases showed a delayed appearance and 1 case suggested a suspect decrease of output; 9 cases were apparently free from pathologic changes of the liver.

After all, I found hepatic disturbance in 38 cases (70%), 45 cases (83%) containing a suspect dysfunction among 54 diabetics.

(ii) Relation of hepatic function to the type of diabetes.

Diabetics are classified in our Clinic by the effect of dietary management characterized by "high carbohydrate, low fat diet," namely, they are divided into Type I which is curable with this dietary treatment and Type II which is obliged to use insulin.

Considering the difference of hepatic disturbance between 2 types, I found a disturbance in 14 cases (54%) showing a normal result and suspect disturbance in 6 cases respectively out of 26 cases of Type I, while a disturbance in 25 cases (89%), suspect in 1 case and normal result in 2 cases among 28 cases of Type II.

Moreover, considering the difference except 4 cases of Type I and 3 cases of Type II who were examined after dietary treatment or insulin injection and 2 cases of readmission, hepatic disturbance was indicated in 50% of 22 cases of Type I and 87% of 23 cases of Type II.

From these facts more hepatic dysfunction was observed in diabetics of Type II than in Type I, showing a significant difference between both types from the statistical point of view.

As above mentioned, 21 cases indicated a characteristic feature showing an increased output and a fall of ratio; that is the amount of output showed a conspicuous increase during the first 4 hours and it was very large in total in spite of a small amount during the last 4 hours, and

TABLE I.

Influence of Dietary Treatment upon Liver Function.

Case (no.)	Type	Sodium santonate test				Azorubin-S test %	Clinical examination				Diet	Duration of dietary treatment (days)
		Appearance time	Output 4 hours	Output 8 hours	Excretory ratio		Blood sugar mgrms. %	Urine sugar grms.	Urine amount c.c.	Urine specific gravity		
1	I	20	71.9	139.4	1.94		87	8.1	1170	1016	F	20
		10	155.2	289.0	1.86		93	17.5	1340	1020	S	
2	I	10	142.6	242.8	1.70	12.3	122	35.7	1600	1023	F	15
		10	497.8	622.1	1.25	9.4	128	10.5	1620	1019	S	
10	I	20	51.7	88.9	1.72	22.6	144	7.2	1100	1029	F	17
		10	179.7	302.6	1.68		108	5.7	1000	1030	S	
11	I	20	135.1	217.1	1.60	31.9	148	60.8	2100	1026	F	30
		10	206.0	340.0	1.65	23.5	171	32.0	1920	1024	S	
14	I	10	107.7	161.7	1.50	9.9	176	11.9	1000	1027	F	7
		10	203.3	289.8	1.43	19.4	171	8.3	670	1020	S	
16	I	10	58.5	106.5	1.82		176	28.4	1540	1023	F	15
		10	234.8	307.3	1.31		165	27.9	1560	1019	S	
19	I	20	128.3	170.2	1.33		212	60.6	2400	1025	F	11
		10	83.3	150.9	1.81		184	3.3	1370	1013	S	
21	I	20	104.8	164.0	1.56		238	23.9	1900	1015	F	29
		20	182.4	233.1	1.28			21.3	1650	1020	S	
22	I	10	376.5	432.0	1.15	7.8	252	76.7	1800	1024	F	
		10	229.8	271.0	1.18		160	16.2	1800	1014	S	14
		10	292.1	347.6	1.19	12.2	122	25.2	1940	1014	S	43
23	I	20	192.1	305.5	1.59		256	196.0	3710	1044	F	
		20	296.2	451.2	1.52		234	81.5	2000	1030	S	18
		10	264.9	389.3	1.47		258	43.6	1830	1026	S	38
24	I	10	377.8	560.4	1.48		262	92.1	4130	1030	F	11
		10	398.4	527.9	1.33	9.5	236	24.9	1710	1030	S	
25	I	10	289.8	464.8	1.60	18.8	263	74.7	1150	1033	F	24
		10	186.8	395.4	2.12	17.7	178	3.2	1000	1019	S	
26	I	10	212.8	305.3	1.43	21.7	268	232.4	4570	1033	F	7
		10	127.7	166.7	1.31		123	53.3	1730	1020	S	
30	II	20	211.9	273.1	1.29	30.8	212	108.3	1600	1033	F	11
		20	306.9	397.6	1.30	24.6	178	71.6	1380	1032	S	
38	II	10	347.1	449.0	1.29	24.1	273	238.1	3570	1037	F	10
		10	299.5	333.4	1.11	28.4	174	149.1	2300	1033	S	
40	II	20	333.3	428.9	1.29		280	197.2	3700	1037	F	7
		20	426.6	474.8	1.11			123.8	2130	1032	S	
42	II	20	328.3	360.6	1.10	25.4	294	194.7	3360	1039	F	14
		10	270.1	387.0	1.43	19.1	300	76.2	1920	1033	S	
45	II	10	437.6	509.8	1.16		296	137.5	2500	1049	F	
		10	503.2	554.9	1.10		279	62.9	1370	1042	S	9
		10	257.3	280.4	1.09		198	84.2	2300	1040	S	27
46	II	10	378.3	405.3	1.07	21.2	297	337.3	4370	1035	F	8
		10	358.3	404.1	1.13	18.9	206	162.2	2230	1028	S	
47	II	20	288.9	331.6	1.15	15.6	298	726.2	9400	1041	F	8
		20	325.3	388.8	1.20	16.8	208	321.0	3910	1043	S	
48	II	10	331.8	401.8	1.21	23.0	303	418.9	5100	1040	F	16
		10	287.1	319.4	1.11	18.7		184.9	2430	1045	S	

TABLE I.

(Continued)

Case (no.)	Type	Sodium santonate test				Azorubin-S test %	Clinical examination				Diet	Duration of dietary treatment (days)
		Appearance time	Output 4 hours	Output 8 hours	Excretory ratio		Blood sugar mgrms. %	Urine sugar grms.	Urine amount c.c.	Urine specific gravity		
49	II	10	380.5	406.0	1.07	10.0	304	164.9	2330	1038	F	15
		10	287.0	322.7	1.12		228	143.6	2180	1037	S	
50	II	20	293.4	321.9	1.10	24.0	367	378.1	5400	1037	F	
		20	200.2	246.4	1.23	17.9	315	108.8	1780	1036	S	8
		10	260.6	321.3	1.23		208	96.8	2330	1025	S	22

F...full diet S...standard diet

a fall of excretory ratio was caused as natural consequences showing 1.17 on an average.

It is very interesting to find this notable feature in many diabetics, in view of the fact that I could not observe this up to this day except one case each of hepatitis, peptic ulcer and cancer of the stomach among over 110 cases of other diseases.

Moreover, this feature was indicated in 1 case among 26 cases of Type I in which insulin must be injected after readmission with a complication of otitis media, and in 20 among 28 cases of Type II. Thus, an increased output and a fall of ratio were found with a marked singularity in diabetics, especially of Type II.

On the other hand, this was found in severe diabetics from the clinical observations such as the fasting blood sugar level, the amount of urine sugar, urine output and specific gravity; for example, I found in only one out of 17 cases below 200 mgrms. % of the blood sugar level, in 4 out of 12 cases from 201 to 250 mgms. %, in 16 out of 25 cases above 251 mgrms. % showing 14 out of 17 cases above 280 mgrms. %.

2) Hippuric Acid test.

22% of 33 cases were indicated as pathological, showing a disturbance in 3 among 14 cases of Type I and 4 among 19 cases of Type II.

3) A-S test.

Concerning the excretory function in diabetes, Bowen and others[25] reported that bromsulphalein retention in blood stream was present in 5 out of 23 cases, Nakagawa and Mitani[26] found a dysfunction of the liver in 2 out of 4 cases by A-S test and Shimizu and others[27] also found in all cases except one out of 36 cases by Congored test.

In my series of 43 cases, 28 cases (65%) gave a pathologic result. No significant difference was observed between the two, from the statistical

point of view, showing a positive result in 55.5% of 18 cases of Type I, in 72% of 25 cases of Type II.

The grade of disturbance in both types was as follows: (卌) 2 cases, (卄) 4 cases, (+) 4 cases in Type I; (卌) 4 cases, (卄) 6 cases, (+) 8 cases in Type II.

4) Urobilinogenuria.

Bang[28] found the urobilinogen in the urine almost always in cases in which ketonuria was present, Koga[24] also observed in 53%, containing suspect 83.3%, of 34 cases of pancreatic diabetes. Rabinowitch[29] observed a pathologic increase of the urine urobilinogen in 27.5% of 3000 estimations. Bowen and other investigators,[25] Fukaya and others[30] found positive in 7 cases each out of their 21 and 12 cases respectively. On the contrary, Hetényi[31] recognized no pathologic increase of urobilinogen in all cases out of 14 and 87 cases with and without complication respectively.

In my series of 55 cases, I found 10 positive cases, namely 3 out of 26 cases of Type I, 7 out of 29 cases of Type II.

5) Millon's reaction.

In diabetics an increase of amino-acid or -N was noticed in general by some authors,[32-36].

Tajima[37] found positive and weakly positive Millon's reaction in one each out of 6 cases.

In my series of 48 cases, the percentage of positive Millon's reaction showed 12.5%, containing suspect 15%, giving one positive and 3 suspect out of 22 cases of Type I and 5 positive, 4 suspect out of 26 cases of Type II.

6) Takata's reaction.

There is only one report concerning this reaction in diabetes described by Koga[24] who found 2 positive among 15 diabetics.

While in my series of 49 cases, 27 cases (55%) gave hepatic disturbance. A marked difference of disturbance (the risk-rate 0.38%) was observed between 2 types, showing 32% of 22 cases of Type I and 76% of 27 cases of Type II.

The grade of disturbance in both types was as follows: (卌) 1 case, (+) 6 cases in Type I; (卌) 1 case, (卄) 8 cases, (+) 11 cases in Type II.

Relation of clinical examination, complication and duration of disease to hepatic function.

All 6 tests of hepatic function were performed within several days.

The blood and urine sugar.—In all tests higher percentage of disturbance was seen in the group above 250 mgrms.% of the blood sugar level, 200 grms. of the urine sugar as compared with in below this reading; and especially in both S test and Takata's reaction, a significant difference

of disturbance was found by the statistical study between below and above 250 mgrms.% and 100 grms.

The amount of urine.—There is no marked relation between hepatic function and the amount of urine in A-S test, Urobilinogenuria and Millon's reaction, but in S test and Takata's reaction higher disturbance rate was noticed in the group above 3000 c.c. showing a remarkable difference (the risk-rate 3.4%) in the former test.

The specific gravity.—In tests except Hippuric Acid test, higher percentage of disturbance was observed in cases with high specific gravity above 1031 than in below this, especially in S and A-S test with the statistical significance (the risk-rate 2.1%, 1.8% respectively).

Blood pressure.—In all tests except Takata's reaction, higher disturbance rate was observed in the group below 130 mm. Hg. as compared with in above this reading. In S and A-S test, all among 8 and 7 cases with the reading below 100 mm. Hg. respectively had a pathologic result, showing the statistical significance in the latter (the risk-rate 4.6%).

Body weight.—I found higher percentage of disturbance in the group below 50 kgrms. than in over this reading in other all tests except Hippuric Acid test, especially a nortable difference was observed showing the risk-rate of 3.7%, 2.1% in S test and Takata's reaction respectively.

Duration of diabetes.—In S test, Millon's reaction and Urobilinogenuria, I found higher percentage of disturbance in the group below 3 years, especially in S test with the statistical significance showing the risk-rate of 3.6%, while in cases with the history above 5 years higher percentage was shown in A-S test.

Age.—In all tests higher disturbance rate was indicated in the group below 39 in comparison with in above 40 years of age, however there is no remarkable difference between the age and liver function.

Complication.—In my series I found 8 cases of pulmonary tuberculosis, 1 case of pleurisy, 3 cases of diabetic cataract, 5 cases of diabetic retinitis, 3 cases of neuralgia, 1 case each of splanchnoptosis, Basedow's disease, cholelithiasis, duodenal ulcer, ascariasis, pancreatic tumour, paralysis agitans, carcinomatous peritonitis, nephritis, scabies, edema, carbuncle, 8 cases of hypotension below 100 mm. Hg. and 10 cases of hypertension over 160 mm. Hg. 13 cases possessed together these 2 or 3 complications. In short, I found 19 cases which had no complication, namely, only about one third of 55 cases.

The disturbance rate of each test in cases who had no complication was as follows: 63.2% of 19 cases in S test, 70.6% of 17 cases in A-S test, 47.1% of 17 cases in Takata's reaction, 20% of 15 cases in Hippuric Acid test, 11.8% of 17 cases in Millon's reaction and 5.3% of 19 cases in Urobilinogenuria.

From this result, the group having complication showed higher disturbance rate in S test, Takata's reaction and Urobilinogenuria, while the contrary result was given in A-S test, and no difference between the functional disturbance and existence of complication from the statistical study was observed.

Influence of diabetic treatment upon liver function.

S and A-S test were employed in order to observe this influence, as is shown in Tables I and II.

1) Outline of treatment.

In all patients full diet was given for several days after admission, and during this time the fasting blood sugar, Staub-effect in addition to the daily amount of urine output, sugar and specific gravity were examined. Then they were placed on a standard diet, which was introduced by the late Prof. Yamakawa[38] in 1936. This diet contains 250–350 grms. of carbohydrate, 70–100 grms. of protein, 20–50 grms. of fat and 1600–2200 calories. It has often been reported from our Clinic[39][40] that this high carbohydrate, low fat diet has a marvellous effect on Japanese. As above mentioned, diabetes is divided into 2 types by the effect of this dietary treatment.

In my series there were some cases beyond the standard limits from such various conditions as body weight.

The effect of this dietary management in Type I was as follows. The urine sugar showed 53.9 grms. on a full diet decreased to about 10 grms. or became free from sugar except some cases excreted more or less 25 grms., the fasting blood sugar fell to 145 from 189 mgrms.%, the urine amount to 1460 from 2050 c.c. and the specific gravity to 1020 from 1025 on an average.

On the contrary, in Type II, the urine sugar decreased to 130 from 255 grms., the blood sugar fell to 233 from 278 mgrms.%, the urine amount to 2390 from 4050 c.c. and the specific gravity to 1035 from 1036 on an average. Therefore, insulin was given 30 minutes before each meal, and on discharge from the hospital the urine sugar was negative or slightly positive without insulin except cases left half-way.

2) Influence of dietary treatment.

13 cases of Type I and 10 cases of Type II were examined by S test. In Cases 11, 1, 10, 14 and 16 which were slightly invalid, hepatic dysfunction was all improved showing a recovery to normal from a delayed appearance in the former 3 cases, a decreased output in the latter 4 cases and abnormal ratio in Cases 10 and 16.

In Case 22 which was tested 2 times after giving for 14 and 43 days,

TABLE II.

Influence of Insulin Treatment upon Liver Function.

Case (no.)	Type	Sodium santonate test				Azorubin-S Test %	Clinical examination				Insulin treatment			
		Appearance time	Output 4 hours	Output 8 hours	Excretory ratio		Blood sugar mgrms. %	Urine sugar grms.	Urine amount c.c.	Urine specific gravity	Treatment	Units a day	Total units	Duration (days)
21	I	20	182.4	233.1	1.28			21.3	1650	1020	D	50	450	9
		20	137.1	203.4	1.48			0	1350	1016	D+I			
24	I	10	398.4	527.9	1.33		236	24.9	1730	1030	D	30	150	5
		20	397.6	604.1	1.52		208	45.1	2380	1031	D+I		300	10
		20	307.6	488.1	1.59		202	26.6	1620	1021	D+I			
28	II	10	389.3	492.8	1.27	14.8	174	154.0	3980	1030	Before	50~30	680	16
		10	279.0	396.4	1.42		184	6.0	2080	1033	D+I			
30	II	20	306.9	397.6	1.30	24.6	178	71.6	1380	1032	D	60~50	460	9
		20	322.1	404.4	1.26	19.2	146	9.1	1200	1027	D+I			
34	II	20	298.7	342.4	1.15		248	310.3	5150	1040	Before	60~30	1440	30
		10	493.6	514.2	1.04		204	108.3	2530	1025	D+I			
37	II	20	233.9	712.9	3.05	11.7	268	383.2	4670	1035	Before	30	360	12
		20	264.4	340.6	1.29	15.9	261	61.6	1620	1031	D+I			
38	II	10	299.5	333.4	1.11	28.4	174	149.1	2300	1033	D	50	950	19
		10	460.3	649.8	1.41	20.0	104	10.0	1810	1021	D+I			
39	II	20	275.3	382.7	1.39	15.7	280	188.5	2280	1037	Before	60	2700	45
		30	82.5	184.3	2.23		183	2.1	1240	1018	D+I			
40	II	20	426.6	474.8	1.11			123.8	2130	1032	D	60	900	15
		10	310.7	343.5	1.11			37.8	1720	1020	D+I			
41	II	10	307.3	395.2	1.29	10.2	288	155.9	3250	1036	Before	60~30	600	13
		10	115.9	138.3	1.19		132	10.2	1730	1020	D+I			
42	II	10	270.1	387.0	1.43	19.1	300	76.2	1920	1033	D	60~45	565	12
		10	250.2	320.6	1.28	20.0	258	1.9	1660	1019	D+I			
46	II	20	358.3	404.1	1.13	18.9	206	162.2	2230	1028	D	75~60	660	10
		20	298.3	355.8	1.19	18.3	179	17.6	1860	1022	D+I			
47	II	20	325.3	388.8	1.20	16.8	208	321.0	3910	1043	D	75	600	8
		10	274.5	331.6	1.21	15.7	148	24.9	2310	1024	D+I			
48	II	20	287.1	319.4	1.11	18.7		184.9	2430	1045	D	90~30	1150	15
		10	244.1	350.6	1.44	11.6	166	2.1	860	1023	D+I		1960	29
		20	174.6	274.6	1.57	16.6	118	0	1140	1021	D+I		2380	41
		20	175.6	259.0	1.47		118	0	1070	1024	D+I			
49	II	20	287.0	322.7	1.12		228	143.6	2180	1037	D	60~50	470	8
		30	50.5	117.7	2.21		153	7.1	1100	1028	D+I			
50	II	10	316.3	385.4	1.22	29.2	310	204.6	3120	1036	D	60~30	1210	24
		10	192.7	247.9	1.29	14.5	124	1.5	1960	1019	D+I			
54	II	10	367.7	516.2	1.40		348	154.9	5200	1032	Before	30	300	10
		10	229.1	341.4	1.49		216	18.6	2200	1021	D+I			

D...dietary treatment I...insulin treatment

excretory ratio was gradually improved in each time, the output recovered on the second time though a slight increase was found on the third time.

Case 26 which showed a marked recovery with dietary treatment gave a normal result both times together.

Case 24 showed no remarkable change.

In Case 23 which was severe as diabetes of Type I, a delayed appearance recovered on the third time though the output remarkably increased.

In Case 21 which indicated the level of 20 grms. of the urine sugar without any change, the output became better although excretory ratio decreased.

Case 25 showed an improvement of the output in spite of a rise of excretory ratio.

Case 2 which was a slight invalid showing the level of 120 mgrms.% of the blood sugar, produced a remarkable increase of the output and a fall of ratio.

Case 19 showed a decrease of output, a rise of ratio in spite of a recovery of a delayed appearance becoming rather worse slightly.

After all, in 6 among 13 cases of Type I the liver function was improved and 4 of them recovered to normal.

The following results were obtained in cases of Type II in which a marked improvement was not indicated with dietary management as is shown in Table I.

Cases 42, 49 and 55 showed an improvement of an increased output and a fallen ratio, and the former 2 cases recovered from a delayed appearance.

Cases 38, 45 and 48 indicated an improvement of the output although a slight increase on the second time was found in Case 45 which was tested 2 times, notwithstanding that a ratio decreased slightly in every case.

After all, it seems to me that these 6 cases which showed still a moderate hyperglycemia and excretion of urine sugar after dietary treatment, indicated an improvement of the liver function especially in the former 3 cases.

No remarkable change was found in Case 46.

In 3 cases (Cases 30, 40 and 47) an increased output was caused after dietary treatment.

As to the results of A-S test, 2 cases (Cases 11 and 25) of Type I and 5 cases (Cases 30, 42, 46, 48 and 55) of Type II were improved within the limits of disturbance, 2 cases (Cases 2 and 22) gave the normal limits both times together and 3 cases (Cases 14, 38 and 47) a slight increase of disturbance grade.

3) Influence of insulin treatment upon liver function.

2 cases of Type I and 15 cases of Type II were examined by S test.

Case 21 which became free from sugar after insulin injection for 9 days, showed a recovery from a fallen excretory ratio. Case 24 which was tested 2 times after giving 150, 300 units of insulin, caused a delayed appearance.

In 9 cases (Cases 28, 37, 40, 42, 46, 47, 48, 50 and 54) of Type II, a fall of the blood sugar and decrease of the urine sugar were found, showing the level of 100 mgrms.% and below 20 grms. except 3 cases with that of 200 mgrms.% and of 20, 30 and 60 grms. An improvement of a marked increased output was observed in all cases, a delayed appearance in 2 cases and an excretory ratio in 4 cases though turned from normal to a fall in Case 42.

In Case 30 which was a slight invalid as diabetes of Type II, there was no remarkable change.

Case 38 showed diabetic recovery after insulin injection, while Case 34 was treated without a marked improvement. An increased output accelerated the grade in both cases notwithstanding that a recovery of a delayed appearance in the latter and an improvement of ratio in the former case were observed.

In Cases 39, 41 and 49 which were tested after insulin injection for 45 days (2700 units), 13 days (600 units) and 8 days (470 units) respectively, the blood sugar fell to the level of 100 mgrms.% from that of 200 mgrms.% and the urine sugar decreased within about 10 grms. from the level of 100 grms. In these 3 cases an increased output decreased beyond the normal limits, and in Cases 39 and 49 a marked delay of the appearance and a rise of ratio were observed, increasing the disturbance of detoxicating function.

8 cases of Type II were examined by A-S test. An improvement was observed in 6 cases (Cases 30, 38, 46, 47, 48 and 50) except 2 cases (Cases 37 and 42) which became slightly worse.

Summary and Discussion.

It is easily supposed that in diabetes there are carbohydrate and other metabolic disturbance due to the want of insulin. Löffler[35] attributed a cause of disturbance to aceton body, and Jones[41] to the undernutrition and acidosis.

Each report made by many investigators suggested its own disturbance rate of the liver function as stated above.

This difference of disturbance rate may be explained by whether the test is many or few and its sensitiveness is great or small. On the other hand, several different function of the liver must be applied in general, for each test suggests a result of its own function.

In my series I found hepatic dysfunction in almost all cases (90%) among 55 cases using 6 methods from the point of detoxicating, excretory, gall secretory function and protein metabolism.

As to the grade of disturbance, Koga[24] stated that it is in the latent

condition. In my series it seems that there is not so high grade from the fact that A-S test and Takata's reaction showed (+) in many cases though it is difficult to estimate in S test, and that other 3 tests indicated low percentage of disturbance.

In S test, 21 cases showed an increased output and a fall of ratio. An increased output which I estimated as liver dysfunction has been supposed as hyperfunction of the liver in general and moreover a disturbance of excretory function following production of Santogenin by some laborators.

A-S excretory test was adopted in 18 among these 21 cases, while no unanimous result was obtained between an increase of the output and excretory disturbance though 12 cases gave a pathologic result. Furthermore, Hippuric Acid test which is the same detoxicating function test as S test was performed in 14 cases, and only 2 cases gave a disturbance and 4 cases had a tendency to indicate hyperfunction showing excretion of more or less 70%.

There are following few reports concerning hyperfunction in diabetes clinically; Shimizu and others[27] reported 3 young cases having a tendency of hyperfunction by Congored test in spite of an increase of aceton body in the urine, Hetényi[31] also noticed this by examination of Urobilinogenuria. From these facts it seems to me that the detoxicating function works actively in the hungry time in severe pancreatic diabetes.

On the other hand, as to the relation between the severity of diabetes and hepatic dysfunction, Shimizu and others[27] reported the dysfunction is parallel to diabetic symptom. In my series, this tendency was also observed from the examination of the blood and urine sugar, urine amount and so on.

As to the age, Meyer[42] said the more advanced in the age is, the more marked dysfunction is, but there was no noted relation between the two in my cases.

Concerning complication, Shimizu and others[27] found hepatic disturbance is great without reference to diabetic symptoms in cases with such complications as pulmonary tuberculosis, but there was no significant relationship between them in my series.

As to the duration of disease, Meyer,[42] Shimizu and others[27] indicated that there shows more disturbance in cases with long history; in my series this tendency was found in A-S test, but cases below 3 years gave more disturbance in S test.

It is generally accepted that the high carbohydrate, protein and low fat diet is suitable in liver injury and it was pointed out by Hara and Yazaki[43] that liver disturbance is caused frequently in diabetes by giving restricted carbohydrate diet for long time which has been adopted from old days.

Concerning the influence of the high carbohydrate, low fat diet upon liver function, about one half of my cases were improved in S and A-S test together with an improvement of diabetic metabolism. Namely, it must be recognized that this standard diet is most ideal as dietary management on Japanese.

Moreover, the influence of insulin upon liver function, especially, upon the content of glycogen has been studied by many investigators and its increase was observed generally in diabetes.

In my results about three-fifths of cases in S test, three-fourths in A-S test were improved. However, in 3 cases of Type II, a remarkable disturbance of detoxicating function was observed in spite of a remarkable recovery from diabetic symptoms; and their body weights were below 37 kgrms. as a characteristic common to all and 2 cases of them had such diabetic troubles in the eyes as cataract and retinitis. I could not confirm whether this is due to insulin itself or substance contained in insulin.

Conclusions.

1. The liver disturbance was found in a majority of fifty five diabetics by Sodium Santonate test, Azorubin-S test, Takata's reaction, Hippuric Acid test, Millon's reaction and Urobilinogenuria.

2. An increased output and a fall of excretory ratio in S test is found with a remarkable singularity in diabetics especially of Type II, from this fact we can differenciate both types and whether diabetes is severe or not.

3. Diabetics of Type II showed generally higher disturbance rate than those of Type I, especially in S test and Takata's reaction with a statistical significance.

There is some interrelation between the presence of liver disturbance and diabetic symptoms observed by the blood and urine sugar, urine amount and specific gravity.

4. No relationship of the blood pressure, body weight, age and complication to the hepatic dysfunction was found excepting that cases with hypotension showed high percentage of disturbance in A-S test and emaciated cases in S test and Takata's reaction.

Cases with long history of diabetes gave not always pathologic result, showing high percentage of disturbance in cases under 3 years in S test.

5. The influence of dietary treatment upon liver function was observed in 23 cases by S test, and about one half of them showed an improvement together with that of diabetic metabolism, especially this tendency was remarkable in Type I showing a recovery to normal quickly in 4 cases. The high carbohydrate, low fat diet must be emphasized again as dietary management of diabetes from the point of an improvement of hepatic function.

6. The influence of insulin administration in addition to the standard diet was observed in 17 cases and I found an improvement in about three-fifths of them, though emaciated 3 cases indicated a deterioration.

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Studies on the Coincidence of Diabetes Mellitus with Gall-bladder, Duct and Liver Diseases in Japan.

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By the successful experiment of von Mering and Minkowski, the relationship of pancreas to diabetes is now generally accepted. Since this brilliant work a study of the liver in diabetes mellitus has been neglected, but there is an intimate relation between the liver and pancreas from various points of view.

In foreign countries many authors have reported the coincidence of gall-bladder and duct diseases with diabetes, and some of them emphasized that in diabetes cholecystitis is of prime etiologic importance.

There are, however, very few reports concerning this problem in Japan.

In the present paper I want to report of the frequency and clinical study about this complication and to review the literature.

Cholelithiasis and Catarrhal Jaundice.

A. Cases Reports.

1) Cholelithiasis.

Among 261 cases of diabetes mellitus 5 cases had gall stone, 4 cases of them (Cases 1–4) had the attack of cholelithiasis during the course of diabetes. There was only 1 case of diabetes who had an anamnesis of cholelithiasis. On the other hand, in 175 cases of cholelithiasis 5 cases had glycosuria. I summarized these cases in Table I.

Cases 1 and 2:

In both cases, the duration of diabetes extended over 10 and 30 years respectively and symptoms of gall stone were found fourteen and thirty months previously. During the admission they complained of typical attack of gall stone. The daily amount of urine sugar was below 100 grms., and I confirmed the latter was Type I of diabetes according to the classifica-

TABLE I.

Cases with Cholelithiasis.

No. of case	Sex	Age	Duration		Diabetic symptoms				
			Diabetes	Cholelithiasis	Blood sugar mgrms. % (Staub)	Urine sugar grms.	Urine amount c.c.	Urine specific gravity	Type
1	M	51	10 years	14 months		7~84	2000 ~3000	1010 ~1030	
2	M	65	30 years	2½ months	158 (Neg.)	20~90	700 ~1700	1025 ~1040	I
3	F	61	6 months	7 days	196 (Pos.)	53~80	2300 ~2800	1020 ~1025	I
4	M	55	15 months	1 months	112 (Pos.)	(+)	1500 ~2500	1010 ~1020	I
5	M	54							
6	M	56	5 months	16 years	128 (Pos.)	0~8	1000 ~2500	1015 ~1025	I

tion of our labolatory.*

Case 3:

The first admission (August 6–October 1, 1942).

For the previous 4 months he complained of fatigue and polyuria, moreover, of migraine on the left side extending over 3 weeks before the entrance and glycosuria was found on a routine examination. In spite of the treatment, however, there was no recovery from migraine. On Aug. 6, she was admitted as diabetes in our Clinic.

On a full diet, the 24 hour specimens of urine was 2050 c.c. in volume, the daily urine sugar was 69 grms. on an average, the fasting blood sugar was 252 mgrms.% and Staub-effect was slightly negative. A functional disturbance of the liver by Santsol test was observed although by Azorubin-S was normal.

10 days after admission, she was placed on a diet containing 300 grms. of carbohydrate, 60 grms. of protein, 40 grms. of fat and 1848 calories, and gradually recovered from diabetes showing 10–20 grms. of the urine sugar, 120 mgrms.% of the fasting blood sugar on discharge from the hospital. 2 months after admission she left the hospital under a diagnosis of diabetes of Type I.

The second admission (February 14–March 12, 1943).

After leaving the hospital she has used to visit our Clinic at intervals

* Diabetics were divided into Type I which recovered with dietary treatment characterised by "high carbohydrate, low fat diet", and Type II in which the use of insulin was compulsory.

of about 1 month. She had at that time 20–25 grms. of urine sugar per day.

She had complained of epigastric pain for 1 week before admission. On Feb. 10, her faeces were ash white in colour with the appearance of jaundice. On 11, the urinary output was 2200 c.c. per day. On 12, the fasting blood sugar of 196 mgrms.%. On 14, she was readmitted with a diagnosis of diabetes mellitus and cholelithiasis.

Bilirubin-index was 23. Ether test of blood serum was negative. Takata's reaction of serum positive.

2 weeks after admission, she recovered from jaundice which was the most intense on Feb. 11, but the scleræ were slightly icteric.

On a general diet, the daily amount of urine output was 2500 c.c., specific gravity 1023, sugar 65 grms. on an average.

There was no great difference between these results of experiments before and after the same dietary management as before, and with the administration of insulin she recovered.

Up to the present time she has been under my observation from time to time and the urine sugar was always constant showing 10–15 grms. per day.

Case 4:

The first admission (January 22–March 30, 1941).

He had been a heavy drinker. For about 2 months before admission he had complained of a dull appetite, fatigue, impotence and slight emaciation; sugar was found on a routine examination. On Jan. 22, he entered our Clinic under a diagnosis of diabetes mellitus.

The liver was palpable about 2 fingers' breadth below the costal margin. It was smooth, not firm and the edge was sharp.

No other abnormalities of importance were found in the physical examination. On a full diet taken for 8 days, the daily amount of urine sugar was 6 grms. on an average, the fasting blood sugar was 134 mgrms.%.

For 20 days after taking a general diet, he was placed on a standard diet containing 300 grms. of carbohydrate, 80 grms. of protein, 30 grms. of fat and 1940 calories.

During his remaining stay for about 40 days in the hospital, the diet was increased to 2135 calories with a carbohydrate content of 350 grms. These dietary managements were enough to keep the blood sugar normal and the urine sugar free or slightly positive.

The second admission (April 30–June 13, 1942).

He had complained of fatigue, night sweat, sense of pressure in the right hypochondrium, burning in stomach and dull appetite for about 45 days before readmission. On April early, he found brown urine and itching; on April 12, jaundice. On April 30, he was readmitted with a

diagnosis of gall stone. Neither fever, vomiting nor epigastric pain had been observed.

His skin was of a yellow tan colour, but not definitely jaundiced and the scleræ were decidedly icteric. The liver was palpable about 4 fingers' breadth below the costal margin and was smooth, not firm, the edge was sharp. The spleen and gall-bladder were not palpable. Bilirubin-index was 24. The gall-bladder failed to visualize by intravenous tetrajodphenolphthalein. The liver damage was observed by Santsol and Azorubin-S test and remarkable by Takata's reaction. With the treatment for about 1½ months no jaundice in the skin was found, and no subjective symptoms.

On a full diet during the admission, the urine volume 1500–2500 c.c., specific gravity 1010–1015, urine sugar was negative or slightly positive and the fasting blood sugar ranged from 86 to 136 mgrms.%.

Case 5:

The invasion and its progress of both diseases were not clear. After admission he complained of typical abdominal pain of gall stone. Though sugar was found in the urine on a routine examination, diabetic symptoms were not remarkable.

Case 6:

About 15 years ago he had complained of stomach-ache, vomiting and jaundice and a diagnosis of cholelithiasis was made; 3 years later cholecystectomy was done.

Since that time, he has complained of the pain over the upper abdomen. For about 5 months before he came under my observation he had complained of the slowness to move the right finger, loss of strength, impotence and weakness in sight; he entered our Clinic as diabetes mellitus.

During admission, the daily urine sugar was negative or slightly positive, the fasting blood sugar was 128 mgrms.% and Staub-effect positive. Roentgen-ray examination of the gastro-intestinal tract revealed a duodenal ulcer and cascade stomach.

2) Catarrhal jaundice.

In 261 cases of diabetes I found 1 case which had the chance of the complication of catarrhal jaundice 3 years ago, 3 cases which had anamnesis of jaundice. In 28 cases of catarrhal jaundice no case with glycosuria was found.

Case 1:

1 year before admission, a diagnosis of diabetes was made but he had symptoms of diabetes persisting for 10 years. 3 years ago he suffered from catarrhal jaundice.

Cases 2, 3 and 4:

These 3 cases entered our Clinic under a diagnosis of diabetes. After

the attack of catarrhal jaundice, it was long before the appearance of glycosuria.

B. Review of the Literature and Discussion.

1) Cholelithiasis.

(a) Frequency of complication.

Kraus,[1] in 1884, found the appearance of sugar in the urine in one case which had primary cancer of common duct with gall stone. Gans[2] also found sugar during and directly after the attacks of abdominal pain of cholelithiasis. The publications of some authors[3-7] who studied the coincidence followed them. Jones[8] found gall stone in 22% of 68 cases of diabetes in the present and past illness, and Wilder[9] said in 58 cases of diabetes 16 cases had gall stone by necropsy. Wöhrmann[10] found biliary disease in 24% of 677 cases in the present and past illness and Haug and Wöhrmann[14] in 36% of 175 cases of diabetes. In 1928, Katsch and his co-workers[10-13] emphasized the relation between the two by description of "Diabetes mellitus as second disease" or "Secondary diabetes after cholecystopathy." After this, Wiechmann[15] noticed that only 4.8% of gall stone in 500 cases who had no glycosuria were seen, although 10.3% in 126 cases of diabetes. Molnar[16] found in 323 cases of diabetes 72 cases had symptoms of gall stone. Furthermore, gall-bladder disease was found in 14.8% of 495 cases of diabetes by Strauss[17] and Horwitz,[18] in 26.8% of 265 cases by Becker,[19] in 17% of 114 cases by Bingel.[20]

On the other hand, concerning the frequency of the complication of glycosuria in cholelithiasis, the following reports were shown, Exner[4] found glycosuria in all of 40 cases of gall stone with the exception of 1 case, however, Hernando[21] saw glycosuria in 6.7% of 762 cases of gall stone, Ohly[22] in 3.8% of 428 cases.

While there were the following reports contrary to the probable relation between two diseases; v. Noorden[23] found gall stone in 2.3%, Seegen[24] in only 1.0% of diabetes. According to Kausch[6] a slight trace of sugar in the urine was found in 1 case among 85 cases of gall stone, Zinn[5] in 2 cases among 89 cases, Strauss[17] and Horwitz[18] found glycosuria in 1.5% of 132 cases of diabetes, Naunyn[25] in 0.4% of 250 cases. Moreover, Freund[26] reported that in 20 cases of gall stone only 2 cases showed diabetic blood sugar curve by the administration of glucose.

In closing, it seems to be supported generally that there is the probable direct relation between the two, although there are some authors who report the low percentage of complication.

In Japan, Sasaki and others[27] found anamnesis of gastric cramp, cholelithiasiss, cholecystitis or pancreatitis in 15.5% of 245 cases of insular diabetes, and in about 19% of extra insular and renal diabetes; but there are very few cases bearing testimony to the probable direct relation of gall-

bladder disease to diabetes. Hotta and Esaki[28] reported 1 case of acute cholecystitis complicated by transitory diabetes.

Turning now to my series, I found 5 cases of complicated gall stone among 261 cases of diabetes and 1 case who had anamnesis of gall stone, and in 175 cases of gall stone 5 cases had glycosuria.

Thus, the probable significance of the relation between the liver, gall-bladder, duct diseases and diabetes is animatedly discussed in Europe and America, however in Japan I find only one statistical report mentioned above.

There are many patients in Europe and America who suffer from diabetes and duct diseases. I am able to think by common sense of an accidental coincidence of these two diseases though there may be some relationship. Considering that the coincidence of gastric cancer with ulcer which is few gives us various interesting subjects, I believe this problem is worth studying from the fact that the complication of diabetes and gall-bladder, duct diseases is few in Japan.

Concerning this difference there is the following report described by Matsuo.[29] In diabetic patients cholesterinemia is generally due to the administration of fat diet, and there may be a difference between the fat metabolism of foreigner and that of Japanese.

(b) Mechanism of complication.

Which is prior in the attack of two diseases?

Katsch and his investigators emphasized the priority of gall-bladder disease. Singer[30] found the priority of gall stone in 26 cases, however, that of diabetes in 9 cases. Kestermann[31] reported an interesting case in which a cholecystectomy caused the appearance of glycosuria. Moreover, some authors[33-35] reported cases which recovered from diabetes after operation upon the gall-bladder.

On the contrary, according to Strauss[17] and Horwitz[18] there was a slight transitory disturbance of pancreas function in 18% of cholelithiasis, but it was rare to find sugar in the urine.

After all, judging from these reports the priority of gall-bladder and duct diseases seems to be generally accepted.

As the mechanism of complication the following reports are indicated. First of all, the anatomical relation between the pancreas and the liver, especially the intimate relation of the common-duct and ducts of Wirsung or Santorini, and moreover the head of the pancreas may be suggested. Katsch and his collabolators said that it is possible for infection to invade the pancreas by way of the lymphaticus and the common duct from the gall-bladder and to lead to the occurence of pancreatitis which causes a secondary disturbance of the islands of Langerhans associated with that of

metabolism, and to produce diabetes.

This opinion was supported by Hochhaus[7] and others.[21][30]

Further, as the etiologic factor of complication, Weidenbaum[3] pointed out the liver damage owing to gall stone, Singer[30] emphasized "liver-diabetes" in addition to the above opinion mentioned by Katsch, Chiray[35] reported the dysfunction of the liver cells, Reicher[36] said that in cases which have complained of long lasting cholelithiasis, diabetes may occur in consequence of the increase of the tissues extending from the gall-duct to the duct of Wirsung and the head of the pancreas.

Molnar[16] and Seckel[37] emphasized hypercholesterinemia and the former reported that the infectious disease, the diminution of the sexual-gland function and achylia gastrica take a part in this.

These reports are all based upon the priority of gall stone; while, according to Horwitz[18] the increase of cholesterin in diabetes causes gall stone. There are some authors[16][30] who point out the probable relation of the hereditary and family condition.

In my series of 6 cases, 4 cases (Cases 1–4) showed the priority of diabetes mellitus. In 1 case (Case 6) in which cholecystectomy was done about 15 years ago and it took over 10 years to be complicated by diabetes, and it is impossible to regard the occurence of diabetes as the result of cholelithiasis. After all, no high percentage of the coexistence and no causal dependence between the two were found.

(c) Clinical features of complicated cases and influence of complication upon diabetic symptoms.

Concerning sex-incidence, it has been recognized by some authors[10][16][17][19][37] that the female was more affected than the male.

As to the age, Jones[8] said the average age was 51, Horwitz[18] also said the complication was the most frequently observed in the fifties.

According to Bingel[20] it was seen very frequently in middle aged persons as compared with in young and old. Cases reported by some authors[31-33] were all over 50.

In my series of 6 cases, Sex: male, 5; female, 1; showing that the males predominate. The age ran from 51 to 65 with an average age of 57.

Concerning diabetic symptoms, Haug and Whörmann[14] pointed out that complicated diabetes has no good prognosis, while Molnar[16] reported it has a good prognosis and also Katsch[11] noticed it is not so severe.

As to the clinical observations of my cases, Case 5 was a slight invalid judging from the anamnesis and symptoms. In 4 cases (Cases 2, 3, 4 and 6) the fasting blood sugar showed 158, 196, 112 and 128 mgrms.% and Staub-effect was positive except Case 2.

The highest daily amount of urine sugar was 84, 90, 80, 0 and 8 grms. in Cases 1, 2, 3, 4 and 6 respectively. Moreover, 4 cases (Cases 2, 3, 4 and

6) showed all Type I, indicating that diabetes associated with gall stone has all good prognosis.

In the following 2 cases I found the influence of the complication of gall stone upon diabetes. Case 3 recovered after dietary treatment during the first entrance, showing 120 mgrms.% of the blood sugar, 10–20 grms. of the urine sugar on discharge, and constantly 20–25 grms. after leaving the hospital. While, on readmission with jaundice, the urine sugar increased to 65 grms., the blood sugar to about 200 mgrms.% and insulin was given because no recovery with the same diet as before was found. After the disappearance of jaundice the urine sugar decreased to 10–20 grms. without the use of insulin.

Case 4, during the first admission recovered with dietary management and on discharge from the hospital the urine sugar was negative or slightly positive; on readmission with gall stone he became free from sugar or sometimes positive in the partial urine without diabetic treatment.

In above mentioned 2 cases, in the former the coincidence seems to have a bad effect, however, in the latter a good effect upon diabetic carbohydrate metabolism.

Becker[19] reported the coincidence of gall stone gives no good influence, and Dibold[38] also noticed the attack of jaundice has a bad effect upon diabetic metabolism, though slightly and transitorily in most instances. Steinitz[39][40] found in 4 cases carbohydrate tolerance fell off before the attack of jaundice. Bade[41] also found a bad effect similarly and especially that it is a notable feature to show at times a remarkable loss in weight and positive Takata's reaction in complicated cases. On the other hand, Ercklentz[42] reported a case which had a striking good effect on carbohydrate tolerance before and during the incidence of jaundice extending about 6 weeks and supposed this is due to an increased making of insulin by giving an impulse as a result of the increase of gall-acid to the pancreas itself and hypophysis-interbrain system or nerve-centre of vagus which regulates the blood sugar. Komatsu[43] reported a case recovered from diabetes together with the disappearance of catarrhal jaundice.

After all, it is probable that the attack of gall stone or jaundice has various effects on diabetic symptoms.

(d) Treatment.

Considering that cholelithiasis is probably one of the most important etiologic factors in the appearance of diabetes, surgical intervention in all cases of the gall-bladder disease was indicated as a means of preventing the occurence of diabetes by Jones[8] and Barber.[44] Rathery[33] mentioned, however, surgical treatment is justifiable in all cases of diabetes with gall-bladder disease, while there are many investigators who give no support to this surgical management.

It is generally accepted it is of importance to protect the liver, as Bade[41] and Dibold[38] emphasized the restricted carbohydrate diet is harmful. It has been reported from our labolatory that the high carbohydrate, low fat diet has an excellent effect as diabetic dietary management, and now it must be, therefore, noted here again from the standpoint of the possibility of the complication of gall-bladder and liver diseases.

2) Catarrhal jaundice.

Flaum and others[45] noticed that in the prevalence of catarrhal jaundice, diabetic patients show a tendency to be complicated by jaundice at high percentage. In 224 cases of the male diabetes, 11 cases of catarrhal jaundice were reported by Dibold[38] and among 334 cases of diabetes 27 cases by Steinitz.[39][40] Concerning the priority of the attack of jaundice, diabetes is generally antecident to the occurence of jaundice.[39][40][41][42][45]

In 20 cases in which the labolatory examination had been performed before the attack of jaundice, Flaum[45] supposed, as an etiologic factor, the patients are infected directly at that time when the blood samples are taken from the ear-lobe judging from the incubations time and pointed out diabetics have especially so many chances.

Steinitz[39][40] believed that on account of a failure of diabetic metabolism the liver has a diminished power of resistance against the infectious virus (streptococcus?) which causes jaundice.

As to sex, Dibold[38] noticed complicated 11 cases were all the male.

In my series of 261 cases, I found no case complicated by jaundice in the present illness, 1 case which 3 years ago complicated by jaundice in the course of diabetes and 3 cases which had an anamnesis of jaundice long before the attack of diabetes.

Briefly, no remarkable relation between the two was observed.

Other Liver Diseases.

There is no report which noticed the complication of diabetes mellitus and liver diseases except cholelithiasis and catarrhal jaundice.

Liver diseases brought here are as follows: 31 cases of cancer of the liver, 27 of cancer of the gall duct, 13 of liver cirrhosis, 8 of liver tumour, 7 of liver abscess, 5 of cholangitis and 10 of others. I found the complication in 5 cases out of 261 cases of diabetes.

These cases were summarized in Table II.

The age of these cases ranged from 39 to 57 with an average age of 48, and in most cases the appearance of liver disease was found in the course of diabetes.

In all cases the liver was palpable, jaundice was observed except Case 2.

In 4 cases (Cases 1–4) in which the labolatory examination was done,

TABLE II.

Cases with Liver Diseases.

No. of case	Sex	Age	Diagnosis of liver disease	Duration		Diabetic symptoms				
				Diabetes	Liver disease	Blood sugar mgrms.% (Staub)	Urine sugar grms.	Urine amount c.c.	Urine specific gravity	Type
1	M	39	Liver tumour	3 months	16 days	222 (Neg.)	95 ~326	1300 ~4200	1030 ~1050	I
2	F	52	Cancer of the liver	4 years	recently	423 (Pos.)	120 ~162	2200	1030	I
3	M	45	Liver tumour	recently	20 days	195 (Neg.)	23 ~210	1500 ~3000	1015	I
4	F	57	Cancer of the gall duct	40 days	20 days	179 (Neg.)	0 ~11	500 ~1500	1015 ~1026	I
5	M	45	Cancer of the liver	1 year	10 months			1500	1020	

the fasting blood sugar was 222, 423, 195 and 179 mgrms.% repsectively, with negative Staub-effect except Case 2; the highest value of daily urine sugar was 326, 162, 210 and 11 grms. on a general diet. These cases, however, showed a remarkable drop of urine sugar by the standard diet indicating Type I, though diabetic symptoms are more or less severe as compared with those of cases complicated by gall stone.

CONCLUSIONS.

1. In this paper the frequency of coexistence and clinical observations were presented in 261 cases of diabetes mellitus and 304 cases of gall-bladder, duct and liver diseases in addition to reviewing the literature.

2. No high percentage of the coexistence of cholelithiasis and diabetes and no causal dependence between the two as it has been emphasized in foreign countries were observed. This is probably due to the difference of fat metabolism between Japanese and foreigner.

3. The majority of diabetic patients associated with gall stone is advanced in age and have a good prognosis.

4. It is probable that the incidence of gall stone or jaundice in the course of diabetes has various effects upon diabetic carbohydrate metabolism; that is, I observed 2 cases, the one seems to have a bad effect showing the rise of blood sugar and the increase of urine sugar by the attack of jaundice, the other shows no such bad effect.

5. There is no such relation between diabetes and catarrhal jaundice as the literature shows.

6. The complication of other liver diseases in 5 cases of diabetes which belonged to Type I was noticed.

Briefly, diabetic patients associated with gall-bladder, duct and liver diseases have a good prognosis. This is helpful to the differential diagnosis of diabetes complicated by jaundice of unknown cause.

The high carbohydrate, low fat diet must be emphasized again as dietary management of diabetes, considering the possibility of the complication of liver, gall-bladder and duct diseases.

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Vi coli in Various Kinds of Disease.

By

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In the cases, which might be clinically diagnosed as *ekiri* but neither dysentery bacillus nor ekiri bacillus was not found out, only the coli bacillus has been often detected from the feces. It seemed to us desirable to investigate in such a case whether it is Vi coli* or not. While it was far from having established any causal relationship between the ekiri-like symptoms and Vi coli, we were able to detect Vi coli in some other kinds of disease.

Method: From feces, urine, blood, purulent matter and inflammatory secretion coli strain was tried to isolate by means of Endo's culture medium, and then of a common agar culture. Then it was identified by means of Gram's coloration, movement, ability of decomposing glucose, lactose, saccharose and sulcit, ability of coagulating milk, formation of indole and agglutination towards the coli immune serum.

The antigen analysis of coli strain was done soon after isolating by means of the object glas agglutination towards the Vi-serum of Salmonella typhi and towards the O- and H-factor serum of Salmonella.

Data:

Ekiri: Only one strain was Vi coli among 14 samples, isolated from 14 children suffered from ekiri-like symptoms, from whom neither dysentery bacillus nor ekiri bacillus was not obtainable.

Acute enteritic diseases: 20 adult cases were examined; no Vi coli was found in feces.

Cystitis and pyelitis: No Vi coli was detected in the urine of 8 patients.

Uterus diseases: 27 cases were tested of the cervical secretion, and Vi coli was noted in a single case.

Acute appendicitis: Coli bacillus was isolated from each piece of the gangrenous appendix, 8 in number, and two were Vi coli. In 16 cases

* The notation "Vi coli" indicates the coli strain with the Vi-antigen, i.e. the Vi-Salmonella antigen.

of the appendicitis catarrhalis acuta and the perityphitic abscess 13 coli strains were isolated. Vi coli was obtained from a perityphlitic abscess, but the coli bacillus isolated from the feces of that patient had no Vi-antigen. This case became later worse.

Coli sepsis: We came to observe a case of the sepsis caused by the coli bacillus, but the strain was not Vi coli.

Control experiments: 20 coli strains obtained from healthy persons had no Vi-antigen.

At terminal it must be mentioned Vi coli, above given, 5 in number, almost lost the Vi-antigen about 2 weeks after the isolation. And our Vi coli strain had neither factor of O- nor H-Salmonella antigen, in contrast to the Salmonella antigen, in contrast to the Salmonella coli, found by Kauffmann.[1] About 1 1/2 year had passed after the isolation, when we tried again the antigen analysis of these 5 Vi coli strains. At that test we found that 2 strains of them completely lost the Vi-antigen and newly presented the XXVI-factor of O-Salmonella antigen, which had been never proved. Such change of the antigenic structure is identical with the V→W change of Salmonella typhi.

Summary.

Vi coli was found in various kinds of disease, viz. *ekiri*, endometritis, and gangrenous and non-gangrenous appendicitis, but rather infrequently. The coli bacilli, isolated from healthy person, and those suffered from the acute enteritis, pyelitis or cystitis, and coli sepsis had no Vi-antigen. It seems to be justified to say some significance of Vi coli upon the severeness of illness. Our Vi coli presented the V—W change about 1 1/2 year after the isolation.

The present work was presented before the Tohoku Medical Meeting, Oct. 1946.

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Biochemical Studies on Carbohydrates.

CXVI. Group-specific Substances in Gastric Mucosa.

Third Communication:

Group-specific Carbohydrates from Human Gastric Mucosa.

By

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(Received for publication, May 30, 1948)

The stomachs and small and large intestines of human cadavars, which had been examined in the Institute of Forensic Medicine and the Patho-anatomical Department, were taken as materials. Mucosa was separated and after removing from mucus, extracted with water. The initial crude products were obtained according to the procedure of Sibuya[1] and purified at first by the aid of glacial acetic acid and afterwards, the carbohydrate from AB stomachs excluded, with cupric hydroxide. In the exceptional case the substance was isolated by fractioning by means of trichloracetic acid.

Of the preparations from the various sources above, those from the stomachs are dealt with in the present account. All of the carbohydrates obtained from A, B and O stomachs were found to resemble the corresponding carbohydrate (A group specific) from hog stomachs[1] in composition,[2] but as regards the optical rotation even the A of them did behave dissimilar to this, which is entirely devoid of the rotatory power.[3] The B and O carbohydrates agreed with each other in the latter respect, diverging from the A. It looks significant that the B carbohydrate had much less serological activity than the A.

The yield of the AB carbohydrate was too small for quantitative analysis, but by analogy its composition is assumed similar to the above carbohydrates, particulary if it is a mixture of the A and B. From the initial crude product from the AB stomach mucosa was also separated a glycidamin, that again was inhibitory to isoagglutination of erythrocytes, though in a low grade. The corresponding glycidamin from hog stomachs will be referred to in the experimental part.

Experimental.

Preparation Procedure.

To obtain the initial crude products were followed the processes which Sibuya adapted to prepare a group specific carbohydrate from pig stomachs, namely, (1) separation of the stomach mucosa, (2) extraction with water, (3) removal from the extract of $Ba(OH)_2$-precipitable substances, (4) acidulation by means of acetic acid with subsequent centrifuging off of the appearing precipitates and dialysis, (6) precipitation after condensation. Yields of those phosphorus-containing, biuret-positive products were 1.6 gm. from A (8 stomachs, 453.3 gm. fresh mucosa), 0.6 gm. from B (4 stomachs, 170 gm. fresh mucosa) and 3.25 gm. from O (10 stomachs, 524 gm. fresh mucosa) stomachs.

They were at first removed from phosphorus by treatment with glacial acetic acid:— To 0.5 gm. of the substances was added glycol (in total 20 drops) drop by drop with kneading and then glacial acetic acid (purified, 99%) similarly to a thick solution. Followingly glacial acetic acid was increased to 15 cc., whereby precipitation occurred. The precipitate was centrifuged[4] and further treated again and again as above, employing anhydrous sodium acetate when a centrifuged supernatant liquid was not transparent. It was completely freed from phosphorus (ammonium molybdate test) by 3–5 reprecipitations. Thereupon it was washed with alcohol repeatedly to eliminate acetic acid, and alcohol replaced by ether, which was evaporated off. 0.3 gm. was the mean crop.

The substances still gave biuret reaction. Those other than the one from the AB mucosa were subjected to the following operations. 100 mgm. substance was made into a paste with a few drops of water, water increased to 5 cc. and alkalized with 0.5 cc. of 10% NaOH. Centrifuged. The centrifugate was again treated in a like manner and to the clear extracts combined were added 3 cc. of 0.5 molar solution of cupric sulfate, the mixture stirred up and under agitation 10% NaOH added in drops until the precipitate got coloured cobalt blue. The centrifugate was suspended in 10 cc. water, made distinctly alkaline to thymolblue with 1–2 drops of 10% NaOH, and centrifuged again. After washing many times in this way (When necessary, sodium sulfate was used to obtain a clear supernatant liquid) the renewed washing remained colourless. The final centrifugate was dissolved in 5 cc. water by addition of 10% HCl to plainly acid to thymolblue. A small insoluble part was centrifuged off and the supernatant liquid precipitated with 5 volumes of 96% alcohol. To eliminate copper the precipitate was washed several times with 10 cc. of alcohol mixed with 1–2 drops of 10% HCl, then washed furtheron with plain alcohol and dried. This copper-alkali procedure was repeated as

long as the substance involved the biuret-giving impurity.

To reduce ash, the substance was then treated with glycol (In some cases water was employed instead) and glacial acetic acid in the way described above. The dried substance was light non-coloured powder. Insoluble in water but soluble in dilute acid as well as in dilute alkali. The yields were very small. From the total materials started with were obtained: A, 56 mgm.; B, 22 mgm.; O, 53 mgm.

Only 2 AB stomachs (97 gm. fresh mucosa) were accessible and the initial crude product was not more than 380 mgm. 350 mgm. of it were removed from phosphorus by means of glacial acetic acid as above, and 210 mgm. were given. Further purification with trichloracetic acid was carried out as follows:— The above product was at first extracted with dilute NaOH (10 cc. water+0.5 cc. 10% NaOH) as in the copper-alkali procedure.[5] The centrifuged supernatant liquids (transparent, 20 cc. in total) were neutralized together with trichloracetic acid (crystals) and additional 1 gm. of this deproteinizing agent added, whereby precipitates appeared. Stood for 30 minutes and centrifuged. From the clear[6] supernatant liquids the carbohydrate was thrown down with 80 cc. of acetone and a little anhydrous sodium acetate. It was washed with alcohol and dried in a vacuum desiccator (P_2O_5). Yield, 7 mgm. The non-coloured product showed similar solubility as the A, B and O carbohydrates. The protein precipitates by trichloracetic acid were washed and dried as above. They (118 mgm.) were hardly soluble in water and showed no inhibition to isoagglutination of erythrocytes. From the substances taken up in glacial acetic acid, the acetone-insoluble were precipitated. After washing with acetone and drying, the precipitate was removed from phosphorus by following the procedure of Utûsi,[7] namely:— It was dissolved in 5 cc. of water by means of the saturated $Ba(OH)_2$, added till pH 10.0 (3 drops were used), and after centrifuging off the part undissolved, the supernatant liquid acidified to pH 2.0 with 1% HCl. The deposit was again rejected by centrifugation. By repetition[8] of such bringing to pH's 10.0 and 2.0, the liquid could be freed from the precipitable substances entirely. After adjustment to pH 4.6 it was dialyzed for 3 days against running water, when Ba disappeared, and then after clarifying by centrifugation and condensation to a small volume, precipitated with 95% alcohol. The deposit was washed with alcohol and dried. 20 mgm. of white powder, which was water-soluble, were the yield.

Qualitative Tests and Biological Assay.

The carbohydrates gave no biuret reaction. The ninhydrin test made on the AB carbohydrate resulted also in negative. But the presence of arginine was proved in the substances. Weak Hiyama reaction[9] was

shown. They contained neither phosphorus nor sphingosin (Thudicum method) nor glycogen (iodine test) nor methylpentose (Rosenthaler test). Molisch reaction, strongly positive. In Osaki and Turumi test for hexosamine, faint direct reaction was given besides the positive indirect, and furthermore hydrolysable sulfur, though in trace, was detected. Sibuya[1] reported that the polysaccharide from hog stomachs has not the direct Osaki-Turumi reaction-giving structure and hydrolysable sulfur. However, he probably took too small amounts of it, because I could demonstrate the positiveness of the tests on a pure preparation[10] of this polysaccharide, which was composed similar to his. Therefore in these connections also, we consider the group carbohydrates from human stomachs to resemble that from pig stomachs.

The phosphorus-free AB substance above from the glacial acetic acid-soluble fraction (a glycidamin) gave the positive biuret reaction. It showed the indirect reaction of Osaki and Turumi but not the direct.

Biologically assayed in our usual method, A carbohydrate was specifically active at a dilution of $1:10^6$ and the B at a dilution of $1:10^5$ (The agglutinin titres of A and B sera used were both 160). The AB carbohydrate did not inhibit the isoagglutination of A erythrocytes at a dilution more than $1:6.4\times10^3$ but inhibited that of B erythrocytes up to $1:10^5$. The glycidamin from the same source exhibited inhibition to the isoagglutination of A and B erythrocytes at dilutions $1:3.2\times10^3$ and $1:6.4\times10^3$ respectively. In these cases, O serum was employed which had titer 80 in both agglutinating A erythrocytes and agglutinating the B.

For the assay the original solutions of the substances were prepared by dissolution of 1 mgm. in 1 cc. of 0.01 n. NaOH with subsequent addition of 1 cc. of $2\times0.85\%$ NaCl solution.

Quantitative Analysis and Optical Measurement.

The methods were our usual ones. The results obtained are embodied in Table I.

The optical rotatory powers were measured by dissolving the substances in 0.01 n. NaOH.

The AB glycidamin contained 10.0% hexosamine and 11.0% hexose expressed as galactose (the orcinol method) and 4.80% ash. It will be worthwhile here to describe the properties of the corresponding glycidamin from pig stomach mucosa. The substance prepared similarly was free from phosphorus and soluble in water and dilute alkali. It gave biuret, Molisch, Hiyama and Osaki-Turumi (both the indirect and direct) reactions. N. 9.38%; hexosamine, 13.5%; galactose, 14.1%. $[\alpha]_D^{21}=-19.64°$. Isoagglutination of A erythrocytes was inhibited specifically by this substance at a dilution of $1:10^5$.

TABLE I.

Composition and Optical Rotation of the Carbohydrates from the Mucosa of Human A, B and O Stomachs.

Composition is expressed in per cent. Figures in parentheses are the calculated on an ash-free basis.

	A carbohydrate	B carbohydrate	O carbohydrate
Nitrogen	4.54 (4.64)	4.45 (4.53)	4.62 (4.72)
Hexosamine	39.83 (40.77)	40.58 (41.37)	40.10 (41.00)
Galactose			
Indole r.	42.37	42.14	41.35
Orcinol r.	40.18	43.57	41.35
Diphenylamine r. I	39.18	41.52	41.35
Mean	40.58 (41.52)	42.41 (43.40)	41.35 (42.28)
Ash	2.34	1.92	2.22
$[\alpha]_D$ (degree)	−25.2 [12°C] (−26.1)	−48.5 [11°C] (−49.4)	−48.0 [14°C] (−49.1)

This work has been supported by a grant from the Education Department through the Grant Committee for Scientific Researches. H. Masamune.

Bibliography and Notes.

1) Sibuya, Tohoku J. Exp. Med., 1949, **50**, 355. Masamune, Sibuya and Tiba, ibid., 1949, **50**, 361.

2) Hexosamine was not identified, but is assumed as glucosamine when analogized from the finding in the group carbohydrate of pig stomach mucosa (cf. 1)).

3) Oh-Uti, K., Tohoku J. Exp. Med., in press.

4) The supernatant liquids were precipitated with acetone and the precipitates set aside to separate anti-hemoisoagglutinative glycidamins.

5) The residue was suspended in 10 cc. of water, shrunk by acidulation with acetic acid (30%; 3 drops) and after centrifugation, washed with changes of alcohol and dried (66 mgm.). This was devoid of anti-hemoisoagglutinative potency.

6) Only opaque liquids separated on centrifugation, when corresponding fractions of human stomachs belonging to other groups and pig stomachs were treated in a like way.

7) Utusi, Tohoku J. Exp. Med., this volume, p. 101.

8) Repeated 5 times.

9) Hiyama, Tohoku J. Exp. Med., in press.

10) This preparation gave weak Hiyama reaction.

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Studies on the Antibiotic Substances from Actinomyces.

(4th Report)

Isolation of Streptomycin.

By

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(Received for publication, November 26, 1949)

In the first step of our systematic study of antagonistic actinomyces, we have isolated 360 antagonistic actinomyces by "streak plate method" (so-called "primary screening"[1]), out of 1,800 strains and we could recognize 6 strains of Str. griseus as streptomycin producing according to their specific antibiotic spectrum and growth ability on the streptomycin containing agar.[2]

Str. griseus No. 346 was cultivated in Thornberry's media by surface culture method, and after the potency reached its maximum, streptomycin was extracted as its hydrochloride by the procedure described by both Cartor et al.[3] and by Vander Brook et al.[4] Crude hydrochloride thus obtained was purified further by chromatographic process and we could obtain almost pure streptomycin hydrochloride (800 u/mg). From this, streptomycin reineckate (decomp. P. 163–166°C) was also crystallized.

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Biochemical Studies on Carbohydrates.

CXVII. The Group-specific Carbohydrates in the Mucosa of Human Small and Large Intestines.

By

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(Received for publication, June 13, 1948)

Group-specific carbohydrates were prepared from human intestines as from stomachs (cf. the foregoing paper[1]). The products were not perfectly pure, mostly containing a trace of phosphorus. However, they were biuret-negative and free from glycogen and cerebrosides and thus looked sufficient to warrant definite conclusion regarding the mutual proportions of sugar components, because the nucleic acid, which was the probable contaminant of the preparations, only reacts very weakly with the usual colorimetric reagents of sugar especially with diphenylamine. The molecular ratio of galactose to hexosamine (presumably glucosamine) was approximately 2:1 in the carbohydrates from small intestines (jejunum and ileum together) belonging to different groups and 4:1 in those from A and O large intestines (colon). The substances were all optically levorotatory, but A carbohydrates were less powerful than the corresponding B and O or than the corresponding O. Of the carbohydrates from large intestines a detailed study of the B was not allowed due to the lack of material, but I assume that it is composed like as the A and O and rotates light like as the O, for the reason by analogy from the relations among the group carbohydrates in gastric juice,[2][3] among those from gastric mucosa[1] and also between the anti-sheep-hemolysin from A urine and the non-seroreactive carbohydrate from B and O urine.[4] With respect to inhibition to erythrocyte isoagglutination, the carbohydrates here dealt with were feeble in comparison with the stomach ones, which was the case in the ones from large intestines in particular. The molecular ratio of galactose to hexosamine appeared thus to decide the intensity of this serological action.

Experimental.

Preparation of the Carbohydrates.

Fresh mucosa collected from small (jejunums and ileums) and large

(colons) intestines, initial crude products and their glacial acetic acid-insoluble fractions, and the final products amounted as listed in Table I.

TABLE I.

Amounts of Fresh Mucosa and Initial and Intermediate Crude Products and Yields of the Group Carbohydrates.

	Group	Number	Mucosa gm.	Initial crude product gm.	Glacial acetic acid-insol. fraction gm.	Group carbohydrate mgm.
Small intestine	A	5	871	2.28	1.72	55
	B	4	524	0.91	0.46	50
	O	4	569	1.27	0.93	30
Large intestine	A	5	335	1.55	1.02	20
	B	4	372	1.01	0.71	9
	O	5	529	1.56	1.02	23

All the carbohydrates obtained were non-coloured, except the one from B colon mucosa. This was free from phosphorus and biuret-giving substances but was tinged brown. Neither glacial acetic acid nor alcohol nor acetone could dissolve out its stain. Activated charcoal could not decolourize its solution in water at acid reaction (at pH 2.0; acidified with dilute HCl), but could at alkaline reaction (at pH 10.0; alkalized with dilute NaOH). Hence, the solution thus decolourized was acidulated and precipitated with alcohol. The recovered substance, washed with alcohol and dried, weighed merely 5 mgm.

The group carbohydrates were sparingly soluble in water, but soluble in dilute alkali and acid.

Qualitative Tests and Biological Assay.

The substances were biuret-negative. Molisch, strongly positive. The indirect Osaki-Turumi reaction was given intensively. The direct reaction was also shown though faint. Hydrolysable sulfur was barely detected in the small intestine-carbohydrates, but was distinctly in the large-intestine-carbohydrates. Traces of phosphorus were present, but sphingosin (Thudicum test), glycogen (iodine test) and methylpentose (Rosenthaler test) were not. Faint Hiyama reaction[5] was shown.

Regarding the inhibition to isohemoagglutination, A and B carbohydrates from small intestines were active at dilutions $1:10^5$ and $1:10^4$ respectively, and those from large intestines at dilutions $1:1.6\times10^4$ and $1:8\times10^3$. Both A and B sera, which were employed, had agglutinin titer 160.

Quantitative Analysis and Optical Measurement.

Table II demonstrates the analytical results.

TABLE II.

Composition of the Group-specific Carbohydrates from Human Small and Large Intestines.

Composition is expressed in per cent. Figures in parentheses are the calculated on an ash-free basis.

Analysis	Small intestine			Large intestine	
	A carbohydrate	B carbohydrate	O carbohydrate	A carbohydrate	O carbohydrate
Nitrogen	4.70 (5.12)	4.86 (5.03)	3.64 (4.62)	1.89 (2.57)	2.35 (2.57)
Hexosamine*	19.5 (21.3)	20.7 (21.4)	15.9 (20.2)	11.7 (15.9)	14.6 (16.0)
Galactose					
Indole r.	42.7	45.0	35.8	47.3	58.7
Orcinol r.	42.9	44.0	34.8	47.3	59.4
Diphenylamine r. I		44.5	34.8		59.4
Mean	42.8 (46.7)	44.5 (46.1)	35.3 (44.8)	47.3 (64.3)	59.2 (65.0)
Ash	8.29	3.48	21.21	26.47	8.94

* Probable glucosamine

The substances rotated D-light in 0.01 n. NaOH as follows:—

The carbohydrates from small intestines:

A, $[\alpha]_D^{20} = \frac{-0.288 \times 100}{1.24 \times 1} = -23.23°$ ($-25.33°$ on an ash-free basis)

B, $[\alpha]_D^{14} = \frac{-0.418 \times 2.0353}{0.0210 \times 1.0032 \times 1} = -40.38°$ ($-41.84°$ on an ash-free basis)

O, $[\alpha]_D^{13} = \frac{-0.248 \times 100}{0.785 \times 1} = -31.59°$ ($-40.09°$ on an ash-free basis)

The carbohydrates from large intestines:

A, $[\alpha]_D^{11} = \frac{-0.164 \times 100}{1.02 \times 1} = -16.08°$ ($-21.88°$ on an ash-free basis)

O, $[\alpha]_D^{14} = \frac{-0.304 \times 100}{0.843 \times 1} = -36.06°$ ($-39.58°$ on an ash-free basis)

We were enabled to carry out this work by a grant from the Grant Committee for Scientific Researches of the Education Department. H. Masamune.

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Prurigo Nodularis.

Preliminary Report.

By

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Prurigo nodularis has been identified by most dermatologists with urticaria perstans verrucosa of Kreibich. Another variety of the latter disease, papulous type of urticaria perstans, however, is quite rare and can hardly be distinguished from a certain type of disseminated neurodermatitis and some prurigo-varieties.

I observed several cases of prurigo nodularis recently and could obtain some interesting results concerning its etiology. The brief summary is following.

In almost every case the onset of the disease ensued from bites by an insect Ensimulium equinum Ihnnaeus, and in one instance even general flare-up phenomena were observed after another bite next year. The watery extract of the insect gave various grade of strong positive reactions on patients' skin by intracutaneous method, and with prolonged subcutaneous injections of the diluted extract beginning with a small amount, relief from itching and hyposensitivity to insect bites were secured. Cutaneous eruptions began to diminish after several injections. The same extract was tested on 17 patients suffering from other skin diseases. Only two reacted with positive reactions, while other 15 showed only very feint reaction or did not at all.

From 3 cases cutaneous nodules were excised for histological examination. In all specimens neuroma-like hypertrophy of nerve bundles was noted in the upper dermal layer by Masson's staining in accord with the observation of Rasch and Pautrier. Control sections from patients with other pruritic dermatoses involving prurigo of Hebra and neurodermatitis failed to reveal increase of nerve bundles, except one case of systemic nevi of lichenoid type with intensive pruritus, although amputation-neuroma-like picture was lacking.

From these findings the author conclude that prurigo nodularis has some causative relationship with insect bites especially bites by Ensimulium, and the hypertrophy of cutaneous nerves resulted from the effect of intensive scratching, while, on the other hand, the nervousness of the patients with abnormal scratch impulse cannot be overlooked when viewed on the fact that abortive form or several isolated nodules occasionally appear in patients with other dermatoses.

The details will be reported in a later issue of this Journal.

e Tohoku Journal of Experimental Medicine, Vol. 51, Nos. 3 & 4, 1949.

Studies on Blood Glycogen.

The 1st Report.

A New Micromethod of Estimation of Blood Glycogen.

By

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At present the most satisfactory procedure for the estimation of blood glycogen is the method of Pflueger.[1] But the blood sample used for the estimation is so much in quantity that it is inconvenient to repeat determinations with such small animals as rabbit. For example, Unshelm[2] uses the blood sample of 10 c.c., Kotschneff[3] and Golandas[4] 1 c.c.

Therefore I have attempted to establish a new micromethod which enables one to determine the glycogen content in the 0.1 c.c. of blood sample.

Method.

Put 0.1 c.c. of the blood sample into a 15 c.c. lidded conical centrifuge tube, which contains 0.5 c.c. of 30% potassium hydroxide, and heat it in a boiling water bath for one hour until the complete digestion of the blood protein is performed. Thereafter add 2 c.c. of absolute alcohol and 0.1 c.c. of 1% sodium sulfate solution; stir thoroughly. After letting it stand overnight in room temperature, centrifuge sharply. Remove the supernatant liquid and wash the precipitate first with 1 c.c. of absolute alcohol and then with 1 c.c. of petroleum ether. After the final washing remove the remaining liquid by heating for a short time on the gas burner. Then hydrolyze the isolated glycogen in 1 c.c. of 5% hydrochloric acid in a boiling water bath for one hour. After cooling neutralize at first with 15% sodium hydroxide and then accurately with 3% solution. The inverted sugar is to be estimated after Fujita-Iwatake.[5]

Although Nerking[6] mentioned the coefficient 0.927 to multiply the inverted sugar content, the constant for my method should be 0.942 ($=0.9\times\frac{100}{95.5}$), while the rate of the recovery of glycogen for my micromethod is 95.5%.

For the estimation in the blood plasma put 0.2 c.c. of the blood sample into the tube which is previously filled with 0.8 c.c. of 3.5% sodium citrate solution. 0.5 c.c. of the supernatant liquid should be used for the estimation in the same manner as blood glycogen.

Discussion.

Alkaline Hydrolysis.

1. Duration of Alkaline Hydrolysis.

Pflueger's[1] original direction for tissue glycogen requires 3 hours heating with KOH. For the blood sample Kotschneff[3] recommends 45 minutes, Golandas[4] 1 hour and Unshelm[2] 2 hours.

Table I shows that recovery of the pure glycogen in aqueous solution is not influenced by heating at 100°C in 30% KOH from 30 minutes to 3 hours. Moreover, the glycogen content in the blood is not affected by alkaline hydrolysis from 1 to 3 hours.

Table I.

Duration of Alkaline Hydrolysis.

	Before heating	After heating (time in hours)			
		0.5	1	2	3
Pure glycogen (mgrm. %)	90	88	89	89	90
Blood glycogen (mgrm. %)		40	45	45	45

It is the reason that the time to be used for alkaline hydrolysis is 1 hour.

2. Temperature of Alkaline Hydrolysis.

Yamakawa[7] reported that glycogen is not so much influenced by autoclaving at 120°C for 30 minutes as by heating at 100°C for 1 hour.

Table II demonstrates that less pure glycogen in aqueous solution or in the blood may be found by autoclaving at 120°C for 30 minutes than by heating at 100°C for 1 hour.

Table II.

Temperature of Alkaline Hydrolysis.

	Heating at 100°C for 1 hour	Autoclaving at 120°C for 0.5 hour
Pure glycogen (mgrm. %)	79	64
Blood glycogen (mgrm. %)	52	45

Therefore it is concluded that heating at 100°C for 1 hour is more

suitable than autoclaving at 120°C for 30 minutes for alkaline hydrolysis.

Concentration of Alcohol.

Pflueger's[1] direction requires 2 volumes of 95% alcohol, while Lawrence and McCance[8] contend that 80% in the mother liquor is necessary to insure the complete precipitation of glycogen. After Good, etc.[9] 50% in the mother liquor is not less effective than any higher concentration. As for the estimation of blood glycogen Kotschneff[3] adopted 2 volumes of 95% alcohol, but Golandas[4] and Unshelm[2] 50% of alcohol in the mother liquor.

According to the concentration of alcohol in the precipitation of glycogen, Table III gives that the 77% alcohol is sufficient for the almost complete precipitation of glycogen from aqueous solution. Table IV

TABLE III.

Concentration of Alcohol and Precipitation of Glycogen (mgrm.%)

No.	Glycogen in aqueous solution	With 77% alcohol	With 50% alcohol
I	93	91	72
II	73	75	65
Average	83	83	69

TABLE IV.

Concentration of Alcohol and Precipitation of Blood Glycogen (mgrm.%)

	No.	With 77% alcohol	With 50% alcohol
Rabbit	I	56	28
	II	48	16
	III	40	10
	Average	48	18
Mankind	I	51	14
	II	19	2
	Average	35	8

shows the same result by the estimation of blood glycogen. Namely the average loss of glycogen from aqueous solution is 17.6% and that from human and rabbit blood 76.2 % in strength of 50% alcohol in the mother liquor.

After Sahyun[10] by this alcohol precipitation glycogen is adsorbed completely on the charcoal and collects at the bottom of the centrifuge tube. Osterberg[11] reported that the precipitated glycogen is carried down mechanically along with the precipitate of sodium sulfate. Therefore it is recognized in these experiments that sodium sulfate is efficient for the precipitation of blood glycogen, as reported by Kotschneff.[3]

Acid Hydrolysis.

Many investigators have studied and reported on hydrolysis of glycogen with acids for the purpose of ascertaining the conditions which insure as nearly complete inversion as possible. As for tissue glycogen the general agreement is that the best condition is boiling at 100°C with 2.2% hydrochloric acid for 3 to 5 hours. But Takahata and Kume[12] contend that hydrolysis of glycogen with 5% or 10% HCl is completed in 10 minutes, while its hydrolysis with 3% is inverted in 2 hours. As for blood glycogen Kotschneff[3] adopted hydrolysis with 2% HCl for 2 to 3 hours, Golandas[4] with 2.2% HCl for 3 hours and Unshelm[2] with 2.5% HCl for 3 hours.

1. Concentration of Acid Hydrolysis.

In the following experiments hydrolysis of glycogen is performed with 5% and 2.5% HCl. The results given in Table V show that its hydrolysis with lower concentration of HCl is not so much complete as with higher concentration.

TABLE V.

Concentration of Acid Hydrolysis.

Concentration of HCl	Glycogen (mgrm.%) after heating at 100°C (time in hours)			
	0.5	1	2	3
5,0%	74	80	88	89
2,5%	59	72	75	79

2. Duration of Acid Hydrolysis.

Data presented in Table VI show that the best condition of hydrolysis of glycogen is boiling at 100°C for 3 hours under water condenser, while hydrolysis of blood glycogen for 1 to 2 hours.

3. Temperature of Acid Hydrolysis.

Yamakawa[7] preferred for acid hydrolysis autoclaving at 120°C for 30 minutes to heating at 100°C for 3 hours.

The results recorded in Table VII show the obtained glycogen by autoclaving at 120°C for 30 minutes is less than by heating at 100°C for 1 hour.

TABLE VI.

Duration of Acid Hydrolysis.

	No.	After heating at 100°C (time in hours)			
		0.5	1	2	3
Pure glycogen (mgrm.%)	I	94	97	104	104
	II	94	88	97	105
	III	78	82	85	85
	Average	89	89	95	98
Blood glycogen (mgrm.%)	I	37	48	49	48
	II	45	51	58	42
	III	11	19	14	16
	Average	31	39	40	35

TABLE VII.

Temperature of Acid Hydrolysis.

	Heating at 100°C for 1 hour	Autoclaving at 120°C for 0.5 hour
Pure glycogen (mgrm.%)	79	72
Blood glycogen (mgrm.%)	52	36

Recovery of Glycogen.

In Table VIII are given the results of a series of determinations that glucose or inverted sugar from glycogen in aqueous solution or in the blood is entirely destroyed by this procedure and that other reducing agents do

TABLE VIII.

Recovery of Reducing Agents (mgrm.%)

	Reducing powder	Residual reduction	Remarks
Glucose	89	2	
Pure glycogen	78	0	Acid hydrolysis only
	97	0	From alkaline hydrolysis
Blood sugar	84	0	
Blood glycogen	63	2	With 77% alcohol
	52	2	
	42	0	
	27	0	With 50% alcohol

TABLE IX.

Recovery of Glycogen (mgrm.%)

	Pure glycogen		Recovery of glycogen		
	Acid hydrolysis only	From alkaline hydrolysis	Blood of rabbit	The same blood with pure glycogen	Pure glycogen
I	83	83	55	136	81
II	71	69	45	112	67
III	64	66	40	100	60
Average	72,7	72,7	46,7	116	69,3 (95,5%)

not lead to erroneous results. Moreover, glycogen is recovered not only from aqueous solution, but also from the blood sample with an average error of 4.5%, and duplicate analysis of the blood sample gives reproducible results (Table IX).

Normal Content of Blood Glycogen.

The normal glycogen content in the blood is quite various. In the human blood Golandas[4] found 6.7 to 12.2 mgrm.%, Sikinami, etc.[13] 8.6 to 13.3 mgrm.%, Gabbe[14] 18 to 30 mgrm.% and Eufinger[15] 70 mgrm.%; in the human plasma Golandas[4] 2.4 to 3.4 mgrm.%, Sikinami, etc.[13] 4.8 to 8.9 mgrm.% and Gabbe[14] 15 to 20 mgrm.%. In the rabbit blood Golandas[4] pointed out 6.4 to 14.4 mgrm.% and Sikinami, etc.[13] 6.6 to 12.4 mgrm.%; in the rabbit plasma the former 3 mgrm.% and the latter 4.4 to 6.2 mgrm.%.

The results obtained by my new micromethod are as follows: the range of the human blood glycogen content is from 30 to 80 mgrm.% (the average value 55 mgrm.%) and that of rabbit from 20 to 80 mgrm.% (the average value 45 mgrm.%). The average value of the plasma glycogen content of rabbit is 36 mgrm.%, while that of the blood glycogen estimated at the same time is 75 mgrm.%.

SUMMARY.

The experiments present a new micromethod which makes it possible to perform the accurate estimation of glycogen in the 0.1 c.c. of blood sample.

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Studies on Blood Glycogen.

The 2nd Report.

Relation Between Blood and Liver Glycogen.

By

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Undoubtedly the liver is the most important organ in carbohydrate metabolism, especially in the assimilation of glycogen from sugar. Some investigators, such as Ohara[1] and Sikinami, etc.,[2] concluded that liver glycogen pours out into the streaming blood to a certain degree under circumstances of nessecity. But studies on the relation between liver and blood glycogen has been scarce.

Therefore the following experiments are performed in order to clarify the relation of liver glycogen to that of the blood.

Method of Experiment.

In the present experiments rabbits were used regularly after 24 hours fasting. The blood glycogen content was estimated by my[3] own micro-method and the blood sugar by Fujita-Iwatake's[4] method. Data presented in these Tables are an average value between two rabbits.

Results.

1. Blood Glycogen and Sugar Content by Intravenous Injection of Glucose or Fructose combined with Various Vitamins.

After Watanabe[5] from our Clinic vitamin C accerelates the ability of the liver to assimilate the glycogen from intravenously injected glucose or fructose, and thereafter Sasao[6] found a similar fact about vitamin K and P. Staub and Golandas[7] recognized that a hyperglycogenemia occurs within one hour after ingestion of glucose more remarkably than after intravenous injection, but not so distinctly in cases of patients suffering from liver injury. On the contrary Sikinami, etc.[2] contend that the blood glycogen increases within one hour after fructose injection in case of liver injury more remarkably than in case of a healthy body.

As shown in Table I the glycogen in the blood is found increased after

intravenous injection of 10% glucose or fructose 10 c.c. per kg. In case of combined use of glucose or fructose with vitamin C, K and P, the blood glycogen content is found remarkably decreased. For example, 100 mgrm. vitamin C (Redoxon " Roche ") per kg. acts strongly at the beginning, but 10 mgrm. vitamin K (Kativ " Takeda ") or 50 mgrm. vitamin P (Hesperin " Takeda ") per kg. keeps on acting to the end. Similarly those vitamins decrease to a certain degree hyperglycemia produced by injection of glucose or fructose.

TABLE I.

Intravenous Injection of Glucose or Fructose Combined with Various Vitamins.

		Before injection	After injection of glucose or fructose (time in hours)					
			0.5	1	2	3	4	5
Control	Blood glycogen (mgrm. %)	64	60	49	61	60	64	61
	Blood sugar (mgrm. %)	119	117	110	103	103	98	106
Glucose	Blood glycogen (mgrm. %)	42	60	51	59	44	47	52
	Blood sugar (mgrm. %)	87	221	171	126	107	90	88
Glucose with vitamin C	Blood glycogen (mgrm. %)	32	22	23	40	38	28	22
	Blood sugar (mgrm. %)	102	247	208	156	111	91	97
Glucose with vitamin K	Blood glycogen (mgrm. %)	69	74	64	65	60	62	68
	Blood sugar (mgrm. %)	110	232	180	110	97	96	96
Glucose with vitamin P	Blood glycogen (mgrm. %)	61	67	63	79	60	59	56
	Blood sugar (mgrm. %)	95	242	179	108	102	96	91
Fructose	Blood glycogen (mgrm. %)	47	43	41	58	44	53	37
	Blood sugar (mgrm. %)	89	240	161	113	101	90	114
Fructose with vitamin C	Blood glycogen (mgrm. %)	48	41	53	45	40	50	40
	Blood sugar (mgrm. %)	98	220	156	133	110	129	112
Fructose with vitamin K	Blood glycogen (mgrm. %)	52	37	35	42	34	46	49
	Blood sugar (mgrm. %)	116	241	187	132	118	103	119
Fructose with vitamin P	Blood glycogen (mgrm. %)	37	40	41	44	48	45	43
	Blood sugar (mgrm. %)	99	250	193	117	105	100	99

2. Blood Glycogen and Sugar Content by Pilocarpin Injection.

According to our former report[8] pilocarpin hyperglycemia takes place by way of compensation of decreased liver glycogen. And we believe it is very probably in the same process that the blood glycogen increases remarkably by adrenalin hyperglycemia, as reported by Ohara.[1]

Table II shows that the blood glycogen and sugar content are found increased by subcutaneous injection of pilocarpin 5 mgrm. per kg. such as adrenalin.

TABLE II.

Pilocarpin Injection.

		Before injection	After injection of pilocarpin (time in hours)		
			1	2	3
Sugar (mgrm. %)	in the blood	138	211	209	205
	in the plasma	131	201	188	188
Glycogen (mgrm. %)	in the blood	66	68	72	103
	in the plasma	30	54	42	79

Therefore it can be concluded that part of liver glycogen is mobilized into the blood circulation.

3. Blood Glycogen and Sugar Content in Case of Liver Injury.

Kurita[9] reported the liver glycogen disappears rapidly during the first 8 hours in case of phosphor injury from peripheral areas of liver lobules, while it decreases at first slowly, but more and more remarkably 8 to 24 hours later by chloroform intoxication, because the latter destroys at first mainly central parts of liver lobules and later peripheral areas. Staub and Golandas[7] found blood glycogen increment in case of phosphor intoxication.

Data presented in Table III show that a slight increase of blood glycogen with notable hyperglycemia is found during the first 8 hours by subcutaneous injection of 0.5% phosphor 0.75 c.c. per kg., while more and more remarkably 8 to 24 hours later by subcutaneous injection of chloroform 0.25 c.c. per kg.

Thus it becomes clearer that dissoluted glycogen in the liver cells pours out into the blood circulation either as glycogen or as sugar.

4. Effect of Glucose-Vitamin C therapy in Case of Liver Injury.

Watanabe[5] contend that the curative effect of glucose in case of liver injury is intensified by means of combination with vitamin C.

Table IV demonstrates that combination of 25% glucose 10 c.c. per kg. with vitamin C 50 mgrm. per kg. checks the increase of blood glycogen

TABLE III.

Liver Injury by Phosphor and Chloroform Intoxication.

		Before injection	After injection of phosphor or chloroform (time in hours)					
			1	3	5	8	12	24
Control	Blood glycogen (mgrm. %)	52	39	50	51	48	56	52
	Blood sugar (mgrm. %)	106	93	92	94	92	86	97
Phosphor	Blood glycogen (mgrm. %)	38	35	44	41	46	46	19
	Blood sugar (mgrm. %)	108	145	134	133	97	93	76
Chloroform	Blood glycogen (mgrm. %)	40	56	34	46	46	57	64
	Blood sugar (mgrm. %)	76	90	119	103	104	98	104

TABLE IV

Injection of Glucose-vitamin C in Case of Liver Injury.

		Before intoxication	8 hours after intoxication	After injection of glucose-vitamin C (time in hours)				
				1	2	3	4	5
Glucose-C after phosphor	Blood glycogen (mgrm. %)	51	51	45	44	42	59	45
	Blood sugar (mgrm. %)	84	72	199	102	82	113	80
Glucose-C after chloroform	Blood glycogen (mgrm. %)	56	39	40	25	31	37	20
	Blood sugar (mgrm. %)	86	91	212	126	85	82	90

in case of phosphor or chloroform intoxication.

Discussion.

The origin of blood glycogen is attributed to that of the liver (Ohara[1]). Moreover Sikinami, etc.[2] explained it from the experimental results that the blood in the hepatic vein is more concentrated in the glycogen content than in other peripheral blood vessels. Similarly Staub and Golandas[7] contend that it is possible that the liver not only pours out glycogen into the blood as such, but also accepts it as such.

According to our experiments it is obvious that the liver glycogen pours out into the blood as such, not only when the assimilation of glycogen in the liver from glucose or fructose takes place in an unusual speed and quantity, but also when the dissolution of liver glycogen occurs prominently either by pilocarpin or by liver injury. When the ability of the liver to keep glycogen is strengthened by means of combination of glucose or fructose with vitamin C, K and P, the blood glycogen content decreases, even if there be liver injury to a certain degree.

SUMMARY.

From our experiments it can be concluded that part of glycogen in the liver pours into the blood as such, if the ability of the liver to keep glycogen is enfeebled, because the mobilization between glycogen and sugar takes place more rapidly and intensely than usual.

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Colloid Lability of Serum in Skin Diseases.

I. Serum Cobalt Reaction.

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Inoue reported a new colloid lability reaction of serum by substituting cobalt for calcium in Weltmann's coagulation reaction and he named it "serum cobalt reaction" (1942).

I examined this reaction on 616 patients with various dermatoses and 57 controls. The results are embodied in Table I.

TABLE I.

Group	Cases	Coagulation-band		
		shortened (%)	normal (%)	Lengthened (%)
Parasitic dermatoses	43	3 (7.0)	34 (79.0)	6 (14.0)
Virus dermatoses	25	2 (8.0)	21 (84.0)	2 (8.0)
Pyodermas	60	10 (16.7)	38 (63.3)	12 (20.0)
Allergic dermatoses	141	31 (22.0)	86 (61.0)	24 (17.0)
Chemical dermatitis	62	7 (11.3)	43 (69.4)	12 (19.3)
Seborrheal dermatoses	52	0 (0)	38 (73.1)	14 (26.9)
Neurodermatitis	29	2 (6.9)	16 (55.2)	11 (37.9)
Other dermatoses	204	15 (7.4)	138 (67.6)	51 (25.0)
Controls	57	0 (0)	57 (100.0)	0 (0)

The pyodermas, allergic dermatoses and chemical dermatitis frequently showed either shortning or lengthening of the coagulation band. In the neurodermatitis and seborrheal dermatoses the coagulation band had the tendency to be lengthened. But in most of the parasitic and virus dermatoses it remained unaltered.

The details given in a forthcoming paper in this Journal.

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Polysaccharides and a Glycidamin in the Tissue of Gastric Cancer.*

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Working on the stomach mucosa of persons (corpses) belonging to different blood groups, Satoh[1] isolated polysaccharides, which coincided with one another in compostion but differed in optical rotation between the one from A stomachs and those from B and O stomachs. He also separated a glycidamin from AB stomachs. From stomach cancers the present writer obtained, by a similar fractionation procedure, carbohydrates and a glycidamin bearing close resemblance to those from normal stomachs but much less active in inhibiting isoagglutination of erythrocytes and, so far as the polysaccharides are concerned, all with one and the same rotatory power.

Experimental.

Preparation Procedure.

Proper tumor tissue was dissected from exstirpated medullary gastric cancers, and, after removing off blood and necrotic matters by washing, weighed and cut into small pieces to preserve in about 5 volumes of 95% alcohol.

238 gm. of the tissue thus dehydrated were obtained from 10 cancers of persons belonging to Group A. The whole batch of this material was masticated, dried in a Faust apparatus at 37°C and pulverized in a mortar. Fibers discarded, the remaining powder amounted to 32 gm. This was subjected to extraction and fractionation as follows:—

1. Extraction. It was agitated with 10 volumes of water for 2 days (room temperature, 5–9°C), using thymol (0.5 cc. of 20% alcoholic solution) as a preservative, and centrifuged. The residue was treated likewise

* 118th Report of Masamune and coworkers' Biochemical Studies on Carbohydrates.

repeatedly (5 times) with decreasing volumes of water (300, 200, 200 and 100 cc.), until no more substance came out. The centrifuged supernatant liquids (neutral, yellowish, turbid, viscous liquids) were fractioned separately.

2. Treatment with baryta. To the extract was added the saturated $Ba(OH)_2$ in drops to cause maximum precipitation (the pH was shifted to 9.0), stood for 4 hours, supplementing the baryta solution in order to maintain the solution at the mentioned pH, and centrifuged.

3. Elimination of acid-insoluble substances. The supernatant liquid was acidified by means of 30% acetic acid to pH 4.5 with addition of about 0.1 gm. of NaCl per 100 cc. of the fluid and the flocculent precipitates centrifuged, washed with a dilute acetic acid (pH, 4.5) and alcohol and dried in vacuo—$Prec_1$.

4. Dialysis. The slightly turbid liquid (thymol added) was dialyzed through cellophane against running water for 3 days. pH of the solution rose thereby to neutral (temperature of running water, 4–7°C).

5. Concentration and precipitation with alcohol. It was then freed from some precipitate which had appeared, distilled under diminished pressure (foaming was avoided with octylalcohol) to about half the volume and after centrifuging off the precipitate newly occurring, further condensed to a syrup. A sufficient volume of absolute alcohol was added to precipitate all the insoluble part, which was then collected and washed with absolute alcohol and dried. The crops from the successive extracts were 360, 330, 203 (from the third and fourth extracts together) and 25 mgm. These products, coloured yellowish, gave Molisch and biuret reactions and contained phosphorus (ammonium molybdate test).

6. Further removal of acid-insoluble substances. They were combined and extracted 4 times with 20 cc. of water. The remainder undissolved (23 mgm.) was dried—Ns_1. The extracts united, which were yellowish and opalescent, were acidified with drop by drop of 30% acetic acid to pH 4.5, kept in an ice chest overnight and centrifuged. The centrifugate was washed with a dilute acetic acid at pH of 4.5 and alcohol and dried ($Prec_2$, 25 mgm.) and the supernatant clear and viscous fluid was neutralized with NaOH, condensed to a syrup, precipitated with alcohol and dried as in 5.

7. Fractioning by means of glacial acetic acid. The final yellowish substance in 6. was kneaded with 1 cc. of glycol to a thick paste and glacial acetic acid (99%) added at first in drops with thorough mixing and then increased to 15 cc. The precipitates were separated by centrifugation, washed with alcohol and dried in vacuo. This operation was repeated twice, when phosphorus was not detected in it anymore, although the positive biuret reaction was given—$Prec_3$. Yield, 423 mgm. To the moth-

er liquids were added 5 volumes of acetone. The third mother liquid became turbid but gave no precipitate. The precipitates from the first and second were washed with acetone and absolute alcohol and dried—$Prec_4$'s. These, amounting in total to 170 mgm. (=155+15 mgm.), contained phosphorus and exhibited Molisch and biuret reactions.

8. Treatment with copper and alkali of $Prec_3$. 250 mgm. of the glacial acetic acid-insoluble fraction were extracted with three 5 cc. portions of water and to the extracts combined were added a few drops of 10% NaOH to pH 9.0. The precipitate was centrifuged and put aside ($Prec_5$). The supernatant liquid, diluted to 20 cc. with water, was strongly alkalized with 2 cc. of 10% NaOH and immediately 5 cc. of 0.5 m. $CuSO_4$ solution added in small portions under agitation. Following centrifugation gave turbid supernatant liquid. Hence, the centrifugate and the liquid were stirred up together with 50 mgm. of sodium sulfate and centrifuged again. The clear liquid having been discarded, the centrifugate was suspended in 20 cc. of water and after addition of 1 cc. of 10% NaOH, shaken and, after further addition of sodium sulfate as above, centrifuged. In this way washing was made once more and the centrifugate was triturated, suspended in 5 cc. of water and dissolved with the least possible quantity of 10% HCl. The insoluble part was rejected by centrifugation and the supernatant liquid precipitated with 5 volumes of absolute alcohol. The greenish precipitate was suspended in 10 cc. of alcohol, and the smallest quantity of 10% HCl requisite to decolourize it was added. The centrifugate was washed 3 times with 10 cc. alcohol containing 2 drops of 10% HCl and many times with plain alcohol to eliminate HCl and dried in a vacuum desiccator. The processes above beginning with the removal of alkali-insoluble substances were carried out 3 times more (decreasing volumes of water and alkali and other agents were employed). To the noncoloured product, which now gave no biuret reaction, was applied the treatment with glycol and glacial acetic acid in 7. without the use of sodium sulfate that it might be freed from inorganic contaminants. The precipitate by glacial acetic acid, when washed with alcohol and dried, weighed 114.5 mgm.—A-group carbohydrate.

9. Separation of a glycidamin from the glacial acetic acid-soluble fraction. $Prec_4$'s were combined and the glacial acetic acid-insoluble substances accompanying in trace was removed by repetition of the procedure 7. They were then taken up in 20 cc. of water (the solution was faintly acid) and the saturated $Ba(OH)_2$ added to neutral at first and, after centrifuging off of some appearing precipitate, further to pH 9.6, whereupon distinct precipitation occurred. The transparent supernatant liquid after centrifugation was acidified to pH 6.0 by means of 10% HCl, when again an abundant precipitate came out. After standing for several hours,

this precipitate was removed by centrifugation. To the supernatant liquid additional HCl added to pH 2.0, and a small precepitate here was also centrifuged off. After another run of the processes, barium was thrown down with a just sufficient quantity of sulfuric acid (2 n.). The centrifuged supernatant liquid was made neutral with NaOH(0.01 n.), condensed by distillation under reduced pressure and precipitated with alcohol. Since the precipitate still contained phosphorus, though minute, it was again subjected to the same treatment. The substance recovered was found free from phosphorus, but largely consisted of ash. On reprecipitation from a solution in 1 cc. of water with methanol and washing several times with this agent, ash was found reduced but was far from being a trace. The product weighed 27.3 mgm., when dried—A-glycidamin. Molisch and biuret tests were positive.

The materials from persons belonging to different blood groups and the carbohydrates prepared therefrom as above amounted as follow:

Group to which individuals belonged	Fresh tumor tissue gm.	Carbohydrate prepared mgm.
A	238	200
B	45.6	38
O	71.6	61

Chemical and Physical Properties of the Group Carbohydrates.

Qualitative and quantitative analyses.

The preparations were not soluble in water, but were soluble in dilute alkali (0.01 n. NaOH) and acid (0.01 n. HCl). They gave no biuret (for protein) and ammonium molybdate (for phosphorus) tests. The presence of a small amount of hydrolysable sulfur was demonstrated even after electrodialysis. Osaki-Turumi test resulted in positive not only by the indirect process but also, though faint, by the direct. Weak Sakaguchi (for arginine) and Hiyama reactions were shown. Molisch reaction was very intensive. Rosenthaler (for methylpentose) and Neuberg and Saneyoshi (for uronic acid) tests were negative. These sequels are all identical to what were observed in the group carbohydrates from normal human stomachs as well as from pigs' (cf. Satoh, T.[1]).

Quantitative figures obtained by our usual methods are embodied in Table I.

Hexosamine, which is analogized to be glucosamine (cf. Masamune, Sibuya and Tiba[2]), and galactose were involved in an equimolecular proportion and both approached 40% of the substances, like as in the carbohydrates from mucosa of normal human and pig stomachs (cf. Satoh, T.[1])

TABLE I.

Composition of the Group Carbohydrates from Gastric Cancers.

(Gr. ch.=group carbohydrate. Figures in parentheses are the calculated on an ash-free basis.)

Analysis	In per cent			In equivalents per equivalent weight		
	Gr. ch. A	Gr. ch. B	Gr. ch. O	Gr. ch. A	Gr. ch. B	Gr. ch. O
Nitrogen	4.28 (4.34)	4.42 (4.58)	4.40 (4.68)	1.31	1.44	1.47
Hexosamine	41.7 (42.2)	39.2 (40.6)	38.3 (40.7)	1.0	1.0	1.0
Galactose						
Indole r.	43.1	40.8	38.8			
Orcinol r.	41.7	40.6	37.5			
Diphenylamine r. I	42.6	39.1	37.2			
Mean	42.5 (43.1)	40.2 (41.6)	38.0 (40.4)	1.01	1.02	0.99
Acetyl	9.87	9.50	9.49	0.99	1.01	1.03
Ash	1.27	3.45	5.97			

and Sibuya[3]). The present carbohydrates resembled these also regarding nitrogen content. In short, in qualitative as well as quantitative chemical aspects, the cancer substances did not diverge from the ones in the normal mucosa, whereas they exhibited remarkable peculiarities in optical rotation and serological activity as will be described below.

Optical Rotation and inhibition of isohemoagglutination.

No distinction of optical rotation was found among the cancer carbohydrates according to the group. The forces lay intermediately between that of the A carbohydrate ($[\alpha]_D$,−26.1°) and those of the B and O ($[\alpha]_D$, −49.4° and −49.1°) from normal human gastric mucosa (cf. Satoh, T.[1]). Solutions for the measurement were prepared by using dilute alkali (0.01～0.01×10/14 n. NaOH) as a solvent.

Group A: $[\alpha]_D^{13}=\frac{-0.465\times 1.4}{0.0163\times 1}=-39.94°$ (—40.45° on an ash-free basis)

Group B: $[\alpha]_D^{13.7}=\frac{-0.413\times 1}{0.0104\times 1}=-39.71°$ (—41.13° on an ash-free basis)

Group O: $[\alpha]_D^{13}=\frac{-0.414\times 1.4}{0.0155\times 1}=-37.39°$ (—39.76° on an ash-free basis)

To make a further comparison, the writer prepared the group carbohydrate of pig stomach mucosa in a similar manner as described. The obtained substance (N 4.40%, glucosamine 38.4%, galactose 39.6%, ash 2.01%) showed, as a 1% solution, no rotation of D-light at all.

Table II illustrates the specific but very low potencies of the cancer

carbohydrates to inhibit erythrocytes from isoagglutination.

TABLE II.

Occurrence of Agglutination of Erythrocytes by Normal Antisera, Treated with the Cancer Carbohydrates.

A and B sera had agglutinin titers 160 and 80 respectively.

Group of cancer carbohydrate	Group of erythrocytes	Dilution of substance						
		0.1% (Original)	×2	×4	×8	×10	×10²	×10³
A	A	−	−	−	−	−	++	+++
	B	+++	+++	+++	+++	+++	+++	+++
B	A	+++	+++	+++	+++	+++	+++	+++
	B	−	−	−	−	+	+	++
O	A	+++	+++	+++	+++	+++	+++	+++
	B	+++	+++	+++	+++	+++	+++	+++

The A and B carbohydrates from the normal human gastric mucosa are active even at dilutions 1: 10^6 and 1: 10^5 respectively (cf. Satoh, T.[1]).

For the assay was our usual technique followed, which will be noted here again for convenience' sake:—The original solutions were prepared by dissolving substances in 0.01 n. NaOH and adding an equal volume of 1.7% saline solution. pH's of the solutions ranged from 8.2 to 8.6. To 0.2 cc. of the solutions progressively diluted with physiological saline was added 0.2 cc. of 10 times diluted (with physiological saline) serum and the mixtures were kept in a water thermostat at 37°C. for 2 hours, in an ice chest for several hours and at room temperature for 1 hour in sequence. Then 2 drops of a 2% erythrocyte suspension were added and after 1 hour of standing in the thermostat was examined the occurrence of agglutination.

A-Glycidamin from the Cancers of Patients Belonging to Group A.

The glycidamin from A cancers (v. 9. in Preparation Procedure) was soluble in water and analyzed: N 9.78%, hexosamine 7.99%, galactose 9.53% (indole reaction: 9.64%, orcinol reaction: 9.44%, diphenylamine reaction I: 9.51%), ash 30.8%. $[\alpha]_D^{21}$ was −30.65° (−44.29° on an ash-free basis). It gave positive biuret and Molisch tests. Phosphorus was not detected. The direct Osaki-Turumi reaction was shown though faint. The indirect was distinct.

The substance definitely possessed A specific anti-hemoagglutinative power, but was active only up to a dilution of 1: 400 in contradistinction to the corresponding glycidamin (AB) from the normal stomachs, although

both are similar in composition. The latter inhibits the agglutination of A erythrocytes at a dilution of $1:3.2\times10^3$ and that of B erythrocytes at a dilution of $1:6.4\times10^3$ (cf. Satoh, T.[1]).

Is Mucoitin Present in Gastric Cancers?

Takahasi and Yasuoka[4] isolated mucoitin from the mucosa of pig stomachs. It is uncertain at present whether or not this polysaccharide exists in the mucosa of normal human stomachs, but, on the present occasion, the writer tested the residue unextracted and fractions ($Prec_1$, Ns_1, $Prec_2$ and $Prec_5$ in the above) of cancer for glucuronic acid (Neuberg and Saneyoshi method). The results were negative in all. The separated group carbohydrates also did not contain it, as already mentioned.

Discussion.

The present carbohydrates and glycidamin were strikingly less active in the group-specific reaction than the corresponding carbohydrates and glycidamin from the mucosa of normal stomachs. This may be deemed as an indication of their low differentiation with respect to the group-characteristic structure, probably like the corresponding substances in embryos. This assumption is supported by the fact that, in optical rotation, the present A carbohydrate does not differ from the present B and O, contrary to the case of the corresponding normal carbohydrates. In such a sence, the writer calls them "immature compounds." The "immaturity" of compounds in cancer must have close connection with the atypical histological structure and malignity of this tissue.

Summary.

1. Carbohydrates and a glycidamin which are group-specific were isolated from gastric cancers.

2. The above group carbohydrates resemble the corresponding carbohydrates from normal human gastric mucosa in qualitative as well as quantitative chemical composition, but differ from these regarding the strength of optical rotation and anti-hemoagglutinative potency. The writer ascribed the discrepancies to the "immaturity" of those substances.

3. The glycidamin from gastric cancers also showed a relationship like above with the corresponding glycidamin from the normal human gastric mucosa.

4. Mucoitin was not proved present in most of the cancer fractions, that are conceivable to contain it if present.

The costs of this work were defrayed by a grant from the Grant Committee for Scientific Researches of the Education Department, which is gratefully

acknowledged. H. Masamune.

References.

1) Satoh, T., Tohoku J. Exp. Med., this volume, p. 275.
2) Masamune, Sibuya and Tiba, ibid., 1949, **50**, 361.
3) Sibuya, ibid., 1949, **50**, 355.
4) Takahasi and Yasuoka, ibid., 1947, **49**, 65.

The Tohoku Journal of Experimental Medicine, Vol. 51, Nos. 3 & 4, 1949.

Biochemical Studies on Carbohydrates.

CXIX. Hexosamine Compounds in Human Gastric Juice.

Third Communication:

Non-anti-hemoisoagglutinative Carbohydrates in A and B Gastric Juice.

By

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(Received for publication, August 1, 1948)

The carbohydrates N.A.A.C.'s from A and B gastric juice (cf. the first communication[1]) showed close resemblance mutually not only in composition but also in optical property. We incline thus to assume them identical with each other. Although these products exhibited faint anti-isohemoagglutinative potency specifically, it probably owed to imperfect removal of the group carbohydrate A.A.C. during the fractionation. The substances were difficultly soluble in plain water and dilute acid (0.01 n. HCl) but dissolved in dilute alkali.

Experimental.

Analysis and Optical Measurement.

The quantitative-analytical figures of the N.A.A.C.'s, which were obtained by our usual determination methods, are embodied in Table I. The molecular ratios of glucosamine, galactose and sulfuric acid in these polysaccharides approximate to 10: 15: 2, whereas the corresponding ratios in the group carbohydrates were found 10: 10: 1 (cf. the second communication[2]).

The N.A.A.C.'s had similar optical rotations (The substances were dissolved in a dilute Na_2CO_3 solution at pH 8.6):

N.A.A.C. from A gastric juice (pH of the solution, 8.6):

$$[\alpha]_D^{26} = \frac{-0.44 \times 2.9833}{0.0358 \times 1.006 \times 1} = -36.45°.$$

N.A.A.C. from B gastric juice (pH of the solution, 8.6):

$$[\alpha]_D^{19} = \frac{-0.23 \times 2.9911}{0.0184 \times 1.008 \times 1} = -37.09°.$$

Identification of the Monosaccharide Components.

TABLE I.

Composition of N.A.A.C.'s from A and B Human Gastric Juice.

(g. j.=gastric juice)

Analysis	In per cent		In equivalent ratio	
	N.A.A.C. fr. A g.j.	N.A.A.C. fr. B g.j.	N.A.A.C. fr. A g.j.	N.A.A.C. fr. B g.j.
Nitrogen*	3.7	4.35	1.59	1.97
Glucosamine†	29.7	30.9	1.0	1.0
Acetyl‡	7.49	7.49	1.05	1.10
Galactose				
Indole r.§	38.9			
Orcinol r.‖	38.4	39.1		
Diphenylamine r. I§	38.5	39.7		
Mean	38.6	39.4	1.29	1.39
Hydrolysable S¶	1.23	1.23	0.23	0.24
Ash**		2.1		

* Micro Kjeldahl. † Hamasato-Akakura method.
‡ M. Suzuki's modification of Friedrich, Rapoport and Sternberg method.
§ Dische method. ‖ Tillmans and Philippi method simplified by Ozaki.
¶ $BaSO_4$ method under conditions preferred by M. Suzuki. ** Micro analysis.

N.A.A.C. from A gastric juice.

For the purpose the procedure in the 97th information of this series[3] was adapted, namely, the hydrolysate by sulfuric acid was fractioned by means of mercuric oxide, and the hexosamine was identified as pentabenzoate with glucosamine and, as the colorimetric figures in Table I had predicted, the hexose as methylphenylhydrazone with galactose.

From 175 mgm. of the polysaccharide were 54 mgm. of crude hexosamine pentabenzoate obtained. After reprecipitations from methanol, the crystals with a constant melting point of 214.5–215°C. were examined regarding optical rotation and nitrogen content.

$$[\alpha]_D^{14}(\text{in pyridine}) = \frac{0.54 \times 1.4856}{0.0182 \times 0.984 \times 1} = +44.8°.$$

N (micro Kjeldahl).

8.86 mgm. substance, 0.1427 mgm. N.

$(COC_6H_5)_5C_6H_6O_5N$. Found, 2.08%; clac., 2.00%.

Crude galactose methylphenylhydrazone (needles, 48 mgm.) obtained was recrystallized from absolute alcohol four times. By the final recrystallization, the melting point was not raised. Yield, 26 mgm. F.P., 190°C. Mixed melting point test showed no depression.

N (micro Dumas).

3.565 mgm. substance, 0.297 cc. N_2 (20.4°C., 756.99 mm. Hg).

$C_{13}H_{20}O_5N_2$. Found, 9.66%; calc., 9.85%.

N.A.A.C. from B gastric juice.

In this case hydrolysis was made by means of hydrochloric acid.

121 mgm. of the polysaccharide were heated with 10 cc. of 5.5 n. hydrochloric acid in a boiling water-bath for five hours. The appearing dark sediment was centrifuged off. The yellowish supernatant liquid was transferred to a porcelain dish, and concentrated on a water-bath to a syrup and 10 cc. of water added with following filtration. The filtrate was precipitated with 6 cc. of the saturated lead acetate. The centrifuged supernatant fluid was bubbled with H_2S gas, removed from lead sulfide, and after evaporation to about 8 cc., extracted with ether in a continuous extractor for three hours. Ether was aerated off. The liquid obtained was shaken with 7 cc. of 10% NaOH and 1 cc. of benzoylchloride under cooling in ice. The precipitates were washed with water exhaustively to free from alkali and crystallized from 95% alcohol (a small part insoluble in hot alcohol was discarded) and from methanol. The yield at this stage was 38 mgm. and F. P. 208°C. Recrystallized three times from methanol, it melted constant at 215–215.5°C. The amount of substance was reduced to 21 mgm. Crystalline form, thin needle.

N (micro Kjeldahl), 2.09% (5.65 mgm. substance, 0.118 mgm. N).
Calc. for glucosamine pentabenzoate, 2.00%.

$$[\alpha]_D^{12}(\text{in pyridine}) = \frac{0.240 \times 2.9955}{0.0162 \times 0.9830 \times 1} = +45.16°.$$

Summary.

1. N.A.A.C.'s from A and B gastric juice were found identical with each other.

2. It is composed of glucosamine, galactose and sulfuric and acetic acid in molecular ratios of nearly 10:15:2:10.

3. It has the rotation $[\alpha]_D$ of $-36 \sim -37°$.

This work was carried out by a grant through the Grant Committee for Scientific Researches from the Education Department. H. Masamune.

References.

1) Tiba, Teramura and Gotoh, Tohoku J. Exp. Med., 1949, **50**, 287.
2) Tiba, ibid., 1949, **50**, 293.
3) Tiba, ibid., 1949, **50**, 281.

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Demonstration of Langerhans' Cell in the Epidermis.

By

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According to some writers, the mother cell of a malignant melanoma is said to be Langerhans' cell. Masson regarded a pigment nevus as a *gliome langerhansien*. We have been interested in this matter.

The skin, the subject of this paper, was obtained surgically from the palmar surface of thumb of a 52 aged man. As is shown in Figs. 1 & 2, a peculiar dendritic cell was impregnated specifically, by silver tannate method of Hortega, in between the basal layer of the normal epidermis. The Masson's idea will be discussed in the next paper.

Fig. 1.

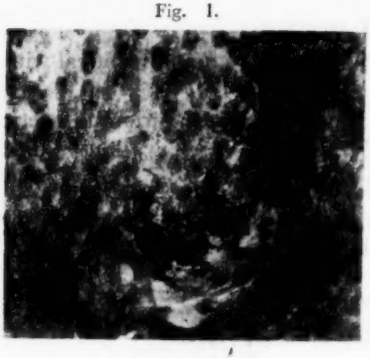
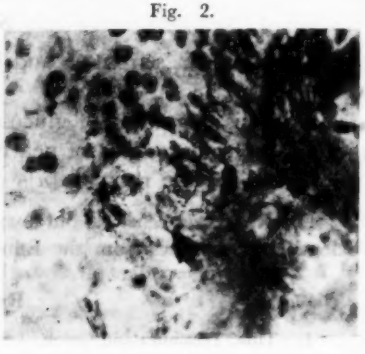


Fig. 2.



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Hexosamine Compounds in Human Gastric Juice.

Fourth Communication:

On the O Gastric Juice and the Digestibility of the Different Group-specific Carbohydrates.*

By

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(Received for publication, August 1, 1948)

"O-specific substance," which Witebsky and Klendshoj[1] separated from gastric juice, gave intensive Molisch reaction but no biuret reaction and showed, on hydrolysis, a reducing power corresponding to 30.3–76.2% glucose as corrected for ash content. It contained 0.0–2.8% N on ash-free basis and was free from phosphorus. The investigators, however, separated the carbohydrates in the gastric juice altogether and hence their preparations are considered to have been mixtures of glacial acetic acid-soluble and insoluble carbohydrates, which, as will be described below, are respectively the O-group-specific and the non-anti-isohemoagglutinative (N.A.A.C.). In the present experiment, I have found that the O-specific carbohydrate in stomach juice has a similar composition as the A- and B-specific from the same source, and resembles B with respect to optical rotation[2] as well as digestibility by the snail liver extract, diverging from A. The non-sero-reactive carbohydrate (N.A.A.C.) from O gastric juice proved identical with the corresponding products from the A and B juice[3] in so far as examined.

Of the total alcohol-insoluble component of O gastric juice, the glycol-insoluble portion containing phosphorus (see the experimental part) was repeatedly shaken with 90% acetic acid to liberate it from phosphorus. The product revealed itself to be a glycidamin containing hexosamine and galactose and hydrolysable sulfur in like equivalent ratios as the non-anti-isoagglutinative carbohydrate above, and gave neither naphthoresorcinol test for hexuronic acid nor aniline acetate test for pentose and hexuronic acid.

*120th report of Masamune and co-workers' Biochemical Studies on Carbohydrates.

In the first communication,[4] we disproved in A and B gastric juice the presence of mucoitinsulfuric acid, which Webster and Komarov[5] insisted in "dissolved" mucin. I could not detect it again in all the fractions, crude as well as purified, of O gastric juice. On this basis it appears justified to reject mucoitinsulfuric acid as a component of stomach juice of at least human beings.

Experimental.

Procedure of Fractioning the Gastric Juice of Persons Belonging to Group O.

The procedure was followed which had been described in the case of A and B gastric juice (the first communication). A typical example will be given below.

From 2400 cc. of pooled gastric juice (pH 2.8) from O patients were obtained 2.9 gm. of the initial alcoholic precipitates with greenish shade (biuret +, Molisch #, naphthoresorcinol —, aniline acetate +, phosphorus #). After separating from the glycol-insoluble part (1.9 gm. in a dry state) (Protein fraction), white powder weighing 580 mgm. was given. Treated with glacial acetic acid, 187 mgm. remained undissolved and from the extract were precipitated 265 mgm.

Purification.

1. The O-specific carbohydrate. The glacial acetic acid-soluble fraction (187 mgm.) was taken up in 10 cc. of water, removed from the insoluble part by repeated centrifugation, and to the transparent supernatant liquid were added 9 volumes of alcohol (95%). The precipitates were dried after washing with alcohol and ether. The substance was freed from the glycol-insoluble contaminants in a like way as above, using glycol (10 cc.) instead of water. 60 cc. of alcohol were added for precipitation. The deposit, washed and dried, still gave biuret reaction, though faint. In order to eliminate the biuret-giving substance, it was electrodialyzed as dissolved in water and followingly shaken, after acidulation,[6] with a chloroform-amylalcohol mixture. But these processes proved not of sufficient use, whereas the manner, next adopted, served the purpose. The solution (1%) was alkalized to pH 8.6 with 5% Na_2CO_3 and shaken for several hours on a machine, and, after addition of dilute acetic acid to pH 4.2 and centrifuging off of the appearing deposit, dialyzed against running water through a cellophane membrane for two days. The dialyzed fluid was then condensed to about 3 cc. and precipitated with acetone. The precipitates were washed with acetone and ether and dried. The white powder obtained was biuret-negative but its amount reached only 64 mgm. It was soluble in water.

2. The glacial acetic acid-insoluble carbohydrate (N.A.A.C.). This was purified in the method employed for the corresponding carbohydrates of the gastric juice from secretors belonging to A and B groups. The crude fraction was found to have already changed difficultly soluble as when pure. Hence, the least requisite quantity of Na_2CO_3 was used each time to redissolve it in water[7] in the course of purification. From 260 mgm. of the crude fraction were produced 100 mgm. of white powder.

3. Glycidamin. 440 mgm. of the protein fraction (the glycol-insoluble fraction) (ammonium molybdate test for phosphorus +, aniline acetate test for pentose +, silver test for purine +, naphthoresorcinol test for hexuronic acid −) were kneaded well in 5 cc. of water and 75 cc. of glacial acetic acid (96%) added in small portions under agitation. The mixture was shaken furtheron on an apparatus for three days and centrifuged. The centrifugate was washed with alcohol and ether and dried. Crop, 290 mgm. The treatment with 90% acetic acid with subsequent washing and drying was repeated more four times. The final product amounted to 120 mgm. It was scarcely soluble in plain water, but gave a solution, though turbid, with sodium carbonate, and precipitated from the solution on acidification to pH 3.8 with acetic acid. It was insoluble in organic solvents including glycol and glacial acetic acid.

Chemical and Optical Properties of the Substances Separated Above.

Qualitative tests.

The O-group carbohydrate. Biüret and ninhydrin reactions were not given. The tests for histidine (diazo), phosphorus, hexuronic acid (naphthoresorcinol), pentose (aniline acetate) and methylpentose (Rosenthaler, Oshima-Tollens) resulted also in negative. Traces of arginine (Sakaguchi) and tyrosine (Millon) were detected. Intensive Molisch reaction was shown. Osaki and Turumi test: the indirect reaction was distinctly and the direct faintly positive. Hiyama test,[8] positive.

N.A.A.C. The results were similar to above in those qualitative tests.

Glycidamin. Biuret, Molisch and Osaki-Turumi (the direct as well as the indirect) tests were all positive. Phosphorus (ammonium molybdate test), pentose (aniline acetate test) hexuronic acid (Neuberg-Saneyoshi), methylpentose (Rosenthaler, Oshima-Tollens) and purine (silver test) were not present.

Quantitative composition.

The analytical figures are embodied in Table I. In composition, the carbohydrates were in accord with the corresponding carbohydrates from A and B gastric juice. The glycidamin was found to contain a carbo-

TABLE I.

Composition of the Polysaccharides and Glycidamin from O Gastric Juice.

Analysis	In per cent			In equivalent ratio		
	O-specific carbohydrate	N.A.A.C.	Glycidamin	O-specific carbohydrate	N.A.A.C.	Glycid-amin
Nitrogen	3.67	4.3	9.8			
Hexosamine	37.1 (Glucosamine)	29.2 (Glucosamine)	11.5	1.0	1.0	1.0
Acetyl	8.43	7.42		0.95	1.06	
Ordinary sugar as galactose	37.7*	40.8*	18.9†	1.01	1.39	1.64
Hydrolysable sulfur	0.69	1.12	0.35	0.10	0.22	0.17
Ash	1.78	5.45				

* The average values by the indole, orcinol and diphenylamine I methods by which the colours deveioped in depths coinciding as expressed as galactose.

† A mean value obtained by the indole, diphenylamine I and α-naphthol I methods which gave the figures 18.3, 19.0 and 19.4% respectively.

hydrate like N.A.A.C. made up of hexosamine, galactose and sulfuric acid in approximate molecular proportions of 1 : 1.5 : 0.2.

Optical rotation.

The O-specific carbohydrate:

$$[\alpha]_D^{22}(\text{in plain water}) = \frac{-1.16 \times 1.3202}{0.0250 \times 1.0032 \times 1} = -61.1°.$$

N.A.A.C.:

The substance was dissolved in water by the aid of Na_2CO_3. pH of the solution was 8.6.

$$[\alpha]_D^{12} = \frac{-0.29 \times 2.9718}{0.0237 \times 1.0002 \times 1} = -36.3°.$$

The rotations of the group carbohydrate and N.A.A.C. from the stomach juice of O persons agree with those of the B-group carbohydrate[2] and of N.A.A.C. from the A as well as B gastric juice[3] respectively.

Identification of Monosaccharide Components of the Polysaccharides from O Gastric Juice.

1. In a like way as in the foregoing investigations[2][3], the present N.A.A.C. was hydrolyzed and glucosamine was separated as pentabenzoate and galactose as methylphenylhydrazone.

114 mgm. of the polysaccharide were taken and 32 mgm. of crude glucosamine pentabenzoate (F.P. 204°C) yielded. This was decreased to 16 mgm. after recrystallization from methanol (F.P. 215°C). No depression was observed in the mixed melting point test.

Optical rotation in pyridine.

$$[\alpha]_D^{10} = \frac{0.22 \times 2.9220}{0.0141 \times 0.9858 \times 1} = +44.9°.$$

N (micro Kjeldahl).

4.84 mgm. substance, 0.102 mgm. N.

$(COC_6H_5)_3C_6H_8O_5N$. Found, 2.11%; calc., 2.00%.

The yield of the crude methylphenylhydrazone (F.P. 179°C) was 30 mgm. The product purified from alcohol (F.P. 188.5°C) amounted to 17 mgm. Its mixture with the corresponding derivative from an authentic preparation of galactose (F.P. 190.5°C) melted at 189°C. N (micro Dumas), 9.38% (calc., 9.85%).

2. Hexosamine in the O-group carbohydrate. It was identified as hydrochloride. 240 mgm. of the polysaccharide was heated for 5 hours in a water-bath with 30 cc. of 5.5 n. HCl containing about 60 mgm. of $SnCl_2$. Through the hydrolysate, when cold, was passed H_2S-gas, then the filtrate evaporated by vacuum distillation, eliminating half way some appearing precipitate by filtration, and 5 cc. of alcohol added to the syrupy condensate. The insoluble substance was centrifuged off and the supernatant liquid placed in an ice-chamber after addition of 1 cc. of acetone. Crystalline deposits (platelets) were collected on a suction funnel and recrystallized from water (The water-insoluble impurity (a trace) was eliminated) by the aid of alcohol and acetone. After another reprecipitation, the product showed the specific rotations in water (17°C) of +91.3° and +71.02° $\left(=\frac{0.81 \times 1.3157}{0.0149 \times 1.007}\right)$ after 10 minutes from dissolution and at equilibrium (4 hours after dissolution) respectively. N (micro Kjeldahl), 6.39% (calc., 6.50%). On heating it began charring at 178°C but did not melt even at 280°C. Thus it was proved to be glucosamine hydrochloride.

Digestion by the Snail Liver Enzyme of the Group Carbohydrates from Gastric Juice.

The enzyme solution was prepared starting with a part of the stock liver porridge that Utusi et al[9] had used, and the procedure of these workers was followed, excepting that the alcoholic enzyme-precipitates were not dried but taken up as such in the acidulated water. For incubation, 3.53, 3.45 and 3.44 mgm. respectively of the group carbohydrates A, B and O were dissolved in 3 cc. of Michaelis' buffer solution at pH 4.5 and mixed with 3 cc. of the enzyme solution and 1 drop of toluene added. From the mixtures was taken out 1 cc. immediately before and at intervals after placing in a thermostat at 37°C to follow up the hydrolysis iodometrically (Macleod and Robison[10]). In the control run where the enzyme solution and the buffer solution containing no substrate were incubated together, no increase of idoine use took place. The amounts of idoine, which were consumed by the substrates A, B and O carbohydrate

before digestion, corresponded severally to 4.8, 5.0 and 5.0% glucose. As is illustrated in Table II, the grades of hydrolysis after 12 hours of

TABLE II.

Increases of Iodine Use by the Group-specific Carbohydrates from Gastric Juice on Incubation with Snail Enzyme.

(Figures show increases of the idoine use as expressed in per cent glucose.)

Group carbohydrate	Time of incubation (day)			
	1/2	1	3	7
A	2.5	5.0	9.1	9.8
B	3.9	5.3	9.3	10.0
O	3.7	5.5	9.5	9.9

incubation diverged plainly between B- and O-sepecific carbohydrates, which coincided in this connection, on one hand and the A-specific on the other. The discrepancy diminished gradually with the progress of digestion and could not be recognized after 7 days from the commencement.

The isoagglutination-inhibitory potency of the A and B carbohydrates, which were active up to a dilution of $1:10^6$, was not impaired within the above duration of digestion. The assay was carried out in our usual method,[11] after dilution of the incubation mixtures first with an equal volume of 1.8% saline and then progressively with physiological saline. The employed A and B sera both had agglutinin titre 160.

The Serological Activity of the O-Specific Carbohydrate.

An immune serum was prepared in the following manner. 15–20 cc. of a 10% suspension of human O-erythrocytes were injected every three days intravenously (V. cervicalis) to a goat. And after 1 week from the 7th injection, the blood was shed and the serum separated.

It agglutinated erythrocytes O and B, when adsorbed in advance with Ae erythrocytes, but did not agglutinate erythrocytes of all the groups when subjected to preliminary adsorption to O or B erythrocytes (Table III). The agglutination of B erythrocytes in the former case is assumed as due either to the preformed anti-B factor in the serum before immunization or more probably to the simultaneous presence of E factor in the B erythrocytes and E-antibody in the immunized serum.

The adsorption above was accomplished by adding to 3 cc. of the serum, 20 times diluted, three 0.05 cc. portions of erythrocytes at intervals of 20 minutes and standing the mixture in air for 3 hours and further in ice for 1 hour. Subsequently the erythrocytes were centrifuged off.

TABLE III.

Agglutination of Human Erythrocytes by Anti-O Immune Goat Serum.

1. After treatment of the serum with O erythrocytes.

Erythrocytes	Dilution of the immune serum							
	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120
Ae	−	−	−	−	−	−	−	−
B	−	−	−	−	−	−	−	−
O	−	−	−	−	−	−	−	−
2. After treatment of the serum with B erythrocytes.								
Ae	−	−	−	−	−	−	−	−
B	−	−	−	−	−	−	−	−
O	±	−	−	−	−	−	−	−
3. After treatment of the serum with Ae erythrocytes.								
Ae	−	−	−	−	−	−	−	−
B	+	+	−	−	−	−	−	−
O	++	+	+	+	−	−	−	−

Table IV demonstrates that all the group-specific carbohydrates from gastric juice inhibit the agglutination of O-erythrocytes by the anti-O serum adsorbed with Ae erythrocytes.

TABLE IV.

Agglutination of O-erythrocytes by the Anti-O Immune Goat Serum Adsorbed with Ae-erythrocytes (dilution, ×20) after Treatment of the Latter with the Group-specific Carbohydrates from Gastric Juice.

Carbohydrate	Dilution of carbohydrate							
	1% Original	×10	$\times 10^2$	$\times 10^3$	$\times 10^4$	$\times 10^5$	$\times 10^6$	$\times 10^7$
A	−	−	+	++	++	+++	+++	+++
B	−	−	∓	+	++	++	+++	+++
O	−	−	+	+	++	++	+++	+++

Here 0.05 cc. of the solutions of the carbohydrates was added to 0.05 cc. of the serum (20 times diluted; adsorbed with Ae erythrocytes) and after 1 hour in a water thermostat at 37°C, one drop of a 2% suspension of O erythrocytes added. The mixtures were again placed in the thermostat and kept for 2 hours to examine the occurrence of agglutination. The result[12] suggests that these specific carbohydrates contain in

their molecule O-structure common to all of them, but the possibility is also allowed that cross reaction takes place between anti-O body in the immune serum and the A and B carbohydrates. The same conjecture is made on the precipitin reaction arising between the carbohydrates and the anti-O serum (Table V) as well as on the inhibitory potency that the carbohydrates exhibited towards the precipitation of O saliva by the anti-O serum (Table VI). In the latter experiment, the saliva from an O-

TABLE V.

Precipitation of Group Carbohydrates from Gastric Juice by the Anti-O Immune Goat Serum.*

Group carbohydrate	Dilution of carbohydrate						
	1% Original	×10	$\times 10^2$	$\times 10^3$	$\times 10^4$	$\times 10^5$	$\times 10^6$
A	++	+	+	±	−	−	−
B	+	+	±	−	−	−	−
O	++	+	+	±	−	−	−

* Undiluted.

TABLE VI.

Precipitation of Saliva* from an O-Secretor by Anti-O Immune Goat Serum† Treated‡ with Group Carbohydrates from Gastric Juice of Secretors.

Group carbohydrate	Dilution of carbohydrate					
	1% Original	×10	$\times 10^2$	$\times 10^3$	$\times 10^4$	$\times 10^5$
A	−	−	−	−	−	−
B	−	−	−	−	−	−
O	−	−	−	−	−	−

* Heated and five times diluted. † Undiluted.

‡ When the immune serum had not been treated with any of the carbohydrates, the dilute saliva was precipitated by the serum at a dilution of 1:40 and the undiluted serum by the saliva at a dilution of 1:64.

secretor was heated for 20 minutes in boiling water and the filtrate diluted five times. The serum was treated with the carbohydrates like as in the experiment illustrated in Table IV, prior to bringing into contact with the saliva.

The Group O carbohydrate naturally did not inhibit the isoagglutination of A and B erythrocytes at all. The procedure employed here was the usual one in this series of investigation.

SUMMARY.

1. Human O gastric juice (of secretors) contains two kinds of polysaccharide, one O group specific and the other non-group-specific.

2. The group-specific carbohydrate above has a composition similar to that of the corresponding carbohydrates in the gastric juice from persons (secretors) belonging to A and B groups, i.e., it is made up of glucosamine, acetic acid, galactose and sulfuric acid in the molecular ratios of 1 : 1 : 1 : 0.1. The proportional molecular number of acetic acid ought to be larger than 1 judging from the sequel of the direct test of Osaki and Turumi, but from analysis the equivalent excess looks minute.

3. The O-specific carbohydrate resembles the group carbohydrate from B gastric juice in optical rotation.

4. The O-group carbohydrate is digestible as readily as the B-group carbohydrate by the snail enzyme but more difficultly than the A group carbohydrate.

5. The O-specific carbohydrate not only inhibits the precipitation of O-saliva (from a secretor) by anti-O immune goat serum and gives precipitin reaction with the immune serum but also inhibits the agglutination of O-erythrocytes by this serum adsorbed with Ae erythrocytes. In these respects, the A and B carbohydrates were similar to the O.

6. N.A.A.C. (the glacial acetic acid-insoluble carbohydrate) in the O gastric juice is regarded identical with the N.A.A.C.'s in A and B gastric juice, which have been proved to be same for their part.

7. Besides the mentioned carbohydrates a glycidamin in the O gastric juice was separated in a phosphorus-free state. Its molecule contains a carbohydrate like N.A.A.C.

8. Neither the alcoholic deposit of the O gastric juice, with which fractionation was started, nor the separated substances gave the reaction of hexuronic acid.

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12) Witebsky and Klendshoj obtained similar results, but their preparations were mixtures of the anti- and non-anti-agglutinative carbohydrates as already mentioned.

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Biochemical Studies on Carbohydrates.

CXXI. On the Direct-Ehrlich Reaction-giving Structure in Glycidamins.

First Report.

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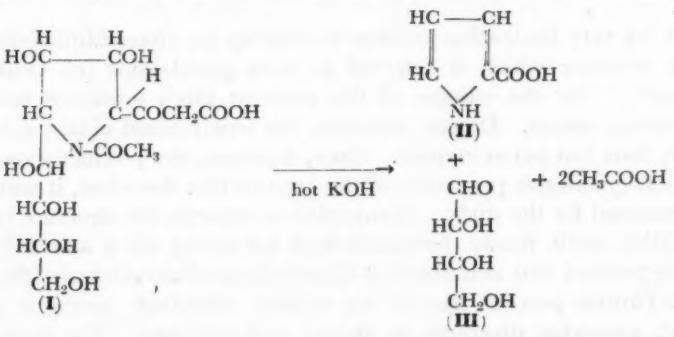
Blix[1] boiled salivary mucin with water and isolated from the extract a crystalline acid ("Kohlenhydrat I") analyzing 42.62–44.70% C, 6.11–6.61% H and 3.68–4.41% N. This substance gave Ehrlich reaction and with dilute $FeCl_3$ a yellow coloration and reduced hot alkaline copper solution. Its neutral equivalent was 381–388 and two molecules of acetic acid were set free when it was heated with *p*-toluenesulfonic acid. On these facts the author assigned it $C_{14}H_{24}NO$ as the most probable formula.

It is a very fascinating problem to clear up the direct-Ehrlich reaction-giving structure which is detected in most glycidamins (cf. Osaki and Turumi[2]). For the solution of this problem Blix's substance makes an appropriate means. Despite attempts, the writer could obtain it only in an oily form but not as crystals. Since, however, the product showed the chemical-qualitative properties of the crystals Blix described, it was taken as a material for the study. Many trials to separate the structure in question failed, until, finally, treatment with hot strong alkali afforded a crystalline product that reacts with *p*-dimethylaminobenzaldehyde (the direct Osaki-Turumi process) like as the mother substance, giving a colour, though somewhat divergent in nuance and spectrum. The spectrum of the colour (purplish) from the product possessed absorption between 530–555 mμ of λ (max. absorp. 550 mμ), whereas, when the Blix's substance is tested, the corresponding colour (red) showes an absorption band between 540–580 mμ (max. absorp. 565 mμ). It showed also a pine shaving test (A pine shaving moistened with conc. hydrochloric acid is dipped in a solution of the substance). A small yield did not allow a farther-going examination so that the salivary mucin itself was next resorted to, and a substance identical (qualitative and mixed melting point tests) with the

above was obtained in a quite sufficient amount and demonstrated to be pyrrole-*α*-carboxylic acid (identified as such and as methylester). That it may have originated in glucosamine or the amino-acid groupings of the mucin was excluded by experimenting with caseinogen and gelatine, which are non-glycidamin simple proteins, and with glucosamine hydrochloride. These proteins gave traces of a substance, which gave the positive pine shaving test for pyrrole and colour reaction with Ehrlich aldehyde, but in the latter reaction the tone and spectrum of the colour differed from those of the colour from pyrrole-*α*-carboxylic acid. Further divergencies of this substance will be described in the experimental part. It looks probable that this pyrrole compound from caseinogen and gelatine was also produced from the mucin but was missed due to its large solubility in chloroform (cf. below). From glucosamine hydrochloride crystals were secured which were in accord with pyrrole-*α*-carboxylic acid in solubility, crystalline form, sublimability and the form of crystals thereby condensing, and the spectrum of the colour that developed by the Ehrlich reaction. But the yield of this probable pyrrole-*α*-carboxylic acid was too small (even less than 1 mgm. from 20 gm. of glucosamine hydrochloride) to assume that, in the experiments on the mucin and the crude Blix's substance, most of pyrrole-*α*-carboxylic acid came from simple glucosamine.

From these observations, following chemism is surmised as regards production of pyrrole-*α*-carboxylic acid (**II**) from the Blix's substance.



To the Blix's substance is here tentatively alloted structure **I**, which satisfactorily explains its properties already quoted. The percentages of C, H and N calculated from it ($C_{13}H_{21}O_{10}N$) amount to 44.4, 6.0 and 4.0, nearly coinciding with the analyses of Blix. Although the equivalent weight (351) according to this formula does not reach the value observed (381–388), the author took too small amounts for accurate determination. As to its direct Ehrlich reaction, cleavage between 2-C and 3-C of the glucosamine chain in **I** and simultaneous dehydration will occur on

heating with the reagent, giving a pyrrole derivative which condenses with the aldehyde. In the course of separating pyrrole-*α*-carboxylic acid the hot strong alkali might have split off the acetyl rests besides. Reduction of hot alkaline copper solution by the Blix's substance is ascribed to *d*-erythrose (**III**) formed from the latter.

Experimental.

Separation of Pyrrole-*α*-carboxylic Acid.

The run started with salivary mucin will be described in full, because the procedure here was typical and the product was most thoroughly examined.

Separation from salivary mucin.

1) The procedure.

Sublingual mucin was prepared from the bull gland according to Hammarsten[3]. It was boiled for 1 hour with 8 volumes of alcohol (95%), washed with hot alcohol and dried in vacuum. 20 gm. of this material were taken up in 200 cc. of 10% KOH and boiled gently in the stream of N_2-gas for 10 hours under reflux. The dark brown digestate removed from some black sediments was diluted to 300 cc. and, under cooling, 10% H_2SO_4 added in small portions to weakly alkaline (pH 7.0–7.4). After evaporating the solution under diminished pressure, the residue was boiled with three 80 cc. portions of 95% alcohol (boiled each time for 30 minutes). These extracts were filtered and distilled together at a low temperature. The remaining dark thick syrup was dissolved in 50 cc. of water (pH of the solution was weakly alkaline), exhausted with ether and then cautiously acidified to pH 2.0 with 20% H_2SO_4. Centrifuged. The centrifugate was washed with a little water and the supernatant liquid and washing united were shaken with five 50 cc. portions of ether. The combined extracts were shaken with about 200 cc. of the saturated $Ba(OH)_2$[4]. Then the watery layer was separated, removed from the excess barium by means of CO_2 and subjected to vacuum distillation to about 100 cc. After decolorization with activated charcoal, it was condensed furtheron to syrupy consistency, eliminating half way some precipitate that appeared. To the remainder was added 96% alcohol as far as precipitation happened and, after one day, precipitates were collected by centrifugation, washed with alcohol and ether and dried in vacuo. The yellowish powder that was given (2.2 gm) proved to contain a considerable amount of barium acetate. Therefore it was dissolved in 50 cc. of water and the insoluble part having been discarded, most of barium

was precipitated out with sulfuric acid (0.5 n.). And the centrifuged supernatant liquid and washing of the centrifugate were united and exhausted with ether. The extract dried with anhydrous sodium sulfate was evaporated in a desiccator (KOH) by means of a jet pump, whereby acetic acid was removed. Brown-stained crystals and resinous substance remained which were stood with chloroform overnight and washed with chloroform on a suction funnel. The crystals obtained undissolved were 25 mgm.

Purification. The crude crystals were dissolved in 5 cc. of water by the aid of the saturated $Ba(OH)_2$[5]. The slightly alkaline solution was treated with charcoal and a just enough quantity of sulfuric acid (1 and 0.1 n. solutions) to precipitate all barium was added and the mixture was treated, immediately without separating off of the precipitates, with ether. The extract was evaporated to about 5 cc. and transferred to a small Erlenmeyer flask. When its volume decreased to 0.5–1.0 cc. on standing in air, crystals in tetragonal (rhombic) and hexagonal plates began to come out. The vessel was stoppered hereupon and full crystallization awaited. Followingly ice-cooled 50% alcohol equal in volume to the content was added and the crystals collected on a suction funnel, washed with the dilute alcohol similarly cooled, and dried in a desiccator ($CaCl_2$) under normal pressure. Yield, 20 mgm.

From two 20 gm. and two 30 gm. portions of the salivary mucin were produced in a like way 83 mgm. of pure crystals altogether.

2) Properties of the product above.

It was readily soluble in methanol, ethanol and ether, and soluble with less ease in water and with difficulty in chloroform.

A red colour developed on treatment with *p*-dimethylaminobenzaldehyde by the direct process of Osaki and Turumi. This colour showed a spectrum with an absorption band from 530 to 555 $m\mu$ of λ (max absorp. near 550 $m\mu$). Pine shaving—a pine shaving was moistened with concentrated hydrochloric acid and immersed in the solution of the product[6], whereby violetred colour appeared—and sodium nitroprusside tests for pyrrole were given. N (Lassaigne) was detected. It did not reduce hot Fehling solution.

By the usual method (an unsealed capillary used) of melting point test, it sintered at 185–190°C and, on further elevation (slow) of temperature, decomposed at 204–205°C under gas evolution. Simultaneously crystals in hexagonal plates and rods condensed at the upper part of the tubelet by sublimation. When the capillary (2 cm.) was sealed according to Ciamician and Silber,[7] it melted at 191.5–192°C under decomposition, and its mixtures with synthetical pyrrole-*α*-carboxylic acid (s. below) and

with the product from " Kohlenhydrat I " (oily product, s. below) showed no depression of melting point. On burning it left no ash.

N (Friedrich).
3.850 mgm. substance, 3.468 cc. 0.01 n. acid neutralized.
$C_5H_5O_2N$. Calc., 12.61%; found, 12.63%.
Neutral equivalent (Pregl).
4.530 mgm. substance, 4.043 cc. 0.01 n. NaOH required for titration.
$C_4H_4N.COOH$. Calc., 111.05; found, 112.05.

By Suzuki's modification[8] of Friedrich, Rapoport and Sternberg method for acetyl determination, the substance gave a volatile acid amounting to 3.98% as acetyl (4.120 mgm. substance, 0.378 cc. 0.01 n. thiosulfate used for titration). This owed probably to carboxyl partially cleft by toluenesulfonic acid.

Methylester was derived according to Schwanert.[9] From 70 mgm. of the product above 125 mgm. of silber salt and 31 mgm. of the ester (long bevelled prisms; F.P., 73°C) were given. The mixed melting point test with the corresponding ester of synthetical pyrrole-α-carboxylic acid (F. P. 73°C, s. below) showed no depression.

N (Friedrich).
5.247 mgm. substance, 4.148 cc. 0.01 n. acid neutralized.
$C_6H_7O_2N$. Calc., 11.2%; found, 11.07%.

Separation from a " Kohlenhydrat I " (Blix) (oily product).

To get the Blix's substance two 30 gm. portions of salivary mucin (submaxillary mucin) were subjected to his preparation procedure at first separately and half way combining the methanol extracts of their fraction, which had been dissolved in boiling water. The oily precipitates from methanol on addition of ether and petroleum ether did not change into crystals. Hence they were dried as such in a vacuum desiccator ($CaCl_2$) and 1.9 gm. of brownish glassy bulk were given. Blix obtained only 30–60 mgm. of the crystalline substance from 40 gm. of the mucin. Therefore the above large yield suggested extreme impureness of the product which, however, was revealed similar in qualitative respects as follows:—

Biuret, ninhydrin, Millon and Molisch tests resulted all in negative. The direct Ehrlich reaction by the Osaki-Turumi technique showed red coloration (max. absorp. 565 mμ). By dilute $FeCl_3$ appeared yellow colour. Hexosamine reaction was demonstrated, though feeble, by the quantitative estimation method of Hamasato and Akakura. The substance reduced hot Fehling solution. In addition, vanilline-hydrochloric acid test of Kobayasi[10] and glacial acetic acid-hydrochloric acid test of the writer[11] gave cherry-red and brownish red colour respectively.

1.8 gm. of the above substance were treated like as the mucin, but

the digestate with 10% KOH was adapted to ether extraction without preliminary fractionation by means of alcohol. 5 mgm. of crystals in hexagonal plates were given. This substance exhibited the direct Ehrlich reaction (Osaki-Turumi process employed). Red coloration with purplish shade took place and absorption extended from about 530 to 555 mμ of λ. In a sealed tubelet it melted at 190°C (decomp.) and the mixed melting point tests with the immediate product from the mucin as well as with synthetical pyrrol-α-carboxylic acid exhibited no depression. In a capillary unsealed, it melted at 204°C (decomp.) and sublimed in part, condensing in crystalline forms with the same appearance as in the case of the other products just mentioned.

N (Friedrich).
3.097 mgm. substance, 2.744 cc. 0.01 n. acid neutralized.
$C_5H_5O_2N$. Calc., 12.61%; found, 12.40%.

Synthesis of Pyrrole-α-carboxylic Acid.

It was prepared with pyrrole and ammonium carbonate according to Ciamician and Silber, but the crude crystals (F.P. in an unsealed tube, 155–158°C) were washed with cold 95% alcohol, whereby the melting point was raised at once to 193–195°C, and further subjected to the procedure adopted to purify the product from the mucin (s. the paragraph "Purification"). The methylester was made by strictly following the method of Schwanert. The preparations both proved authentic.

Trials to See if Pyrrole-α-carboxylic Acid is Obtainable from Gelatine, Caseinogen and Glucosamine Hydrochloride.

An experiment on gelatine.

40 gm. of gelatina alba pulvis E. Merck were taken. The material did not react with *p*-dimethylaminobenzaldehyde at all by the direct as well as indirect-process of Osaki and Turumi. Subjected to the cleavage with KOH and following fractionation described in the case of the mucin, it gave 215 mgm. of a mixture of barium salts, which showed the positive direct-Ehrlich reaction, but after removal of barium, the ether extract left on evaporation only brown resinous substance with few crystal-like granules. On repetition of dissolving in water (the insoluble part separated off), extracting with ether and evaporation, the remainder (about 45 mgm.) looked like before (pyrrole-α-carboxylic acid would crystallize beautifully at this stage). No suitable solvent was found to separate the granules (these were soluble in chloroform) so that the remainder was tested as a whole:— 1) It gave intensive direct Ehrlich reaction (Osaki-Turumi process). Nevertheless, the developing colour (purple) had different

nuance from the one (purplish red) that appears when pyrrole-*α*-carboxylic acid reacts, and its absorption band lay between 540 and 600 mμ of wave length instead of 530–555 mμ in the latter. 2) Pine shaving reaction by the technique described—positive (red coloration). 3) Silver nitrate precipitated a solution of its barium salt as in the case of pyrrol-*α*-carboxylic acid. But the deposit (This gave the characteristic colour by *p*-dimethylaminobenzaldehyde) dissolved rather easily in plain water in contradistinction to the corresponding salt of pyrrole-*α*-carboxylic acid. 4) It was precipitated from water with lead acetate (the precipitates were similar to above regarding the product by Ehrlich reaction), while pyrrole-*α*-carboxylic acid remains in solution. These findings indicated that, from gelatine, a pyrrole compound but not pyrrol-*α*-carboxylic acid is produced, although its exact nature could not be established due to too small a crop.

An experiment on caseinogen.

Hammarsten's caseinogen E. Merck was used. A red colour developed by the *p*-dimethylaminobenzaldehyde reagent (the direct as well as indirect process of Osaki and Turumi) but this reaction was also given by the mere solvent of the reagent. Faint Molisch reaction took place.

Similar operations were applied to 40 gm. of this protein as to the mucin and gelatine. A mixture of barium salts, that was obtained, gave weak Ehrlich reaction and weighed 630 mgm. It was almost free from phosphates (ammonium molybdate test). The evaporated residue of its ether extract consisted only of homogeneous glutinous substance. Ether extraction after fractioning with water and evaporation was repeated as in the foregoing experiment, but it caused no crystallization. The product (65 mgm.) perfectly agreed with the one from gelatine in Ehrlich reaction particularly as to the spectrum of the colour developing hereby, in the pine shaving reaction and in solubility of the silber salt.

An experiment on glucosamine hydrochloride.

20 gm. of glucosamine hydrochloride were treated with 200 cc. of hot 10% KOH with following processes as before. The separated grayish extraordinarily hygroscopic barium-salt mixture amounted to 1.4 gm. It reacted with the *p*-dimethylaminobenzaldehyde reagent (Osaki and Turumi) giving red colour with purplish hue. The spectrum had an absorption band from about 520 to about 570 mμ. Pine shaving reaction in the special way (s. above) and sodium nitroprusside test for pyrrole resulted also in positive. The ether extract of the salt mixture (the water-insoluble part separated off) after removal of barium gave only a brown resinous residue that remained homogeneous on standing, but in the evaporated rest

after extracting it again from water[12] appeared a few hexagonal crystals resembling the crystals of pyrrole-*a*-carboxylic acid. By washing with chloroform the crystals[13] were separated. The total amount did not reach beyond 1 mgm. Heating of it to 180° gave rise to sublimation and condensation into tetragonal and hexagonal crystals as when pyrrole-*a*-carboxylic acid is treated, and these crystals gave the direct Ehrlich reaction with coloration peculiar to this acid (the spectrum showed absorption from 530–555 mμ) and were not precipitated from water by lead acetate but were by silber nitrate after neutralizing with baryta. Lack of substance did not allow further investigation.

Through the Grant Committee of National Research Council a grant was given from the Education Department in aid to us, which is gratefully acknowledged.—Masamune.

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2) Osaki and Turumi, Tohoku J. Exp. Med., 1947, **49**, 11.
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4) When the water layer did not remain alkaline after shaking with the ether extracts, the baryta solution was supplemented.
5) A large volume of water is required to dissolve them without the use of alkali.
6) By this technique the test was negative not only in salivary mucin but also in gelatine and caseinogen.
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12) A part of the residue was found insoluble. This was rejected and the solution treated with charcoal.
13) They were still coloured brown.

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Biochemical Studies on Carbohydrates.

CXXII. The Carbohydrate of Paramucin.

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Several authors[1] engaged in the study of the carbohydrate of paramucin of Mitjukoff. But none of them afforded any definite conclusion as regards its monosaccharide-components, exclusive of Steudel,[1] who isolated glucosamine as the phenylisocyanate derivative from the hydrolysate of the mucin. Thanks to the elaborated recent methods of sugar analysis, the writer could anticipate that it is composed of an amino sugar or amino sugars and galactose in the molecular ratio of 3:2. Based on this fact, identification of the ordinary sugar was easily accomplished by separating its methylphenylhydrazone after hydrolysis of the mucin and following fractionation by our usual procedure.[2] On the other hand, not only glucosamine but also chondrosamine was separated. Here most of the amino acids in the hydrolysates were eliminated by the aid of butanol, as often attempted,[3] and from the mother liquids of glucosamine was obtained chondrosamine on further fractionation with mercuric acetate. The preparation of paramucin employed had similar properties as Mitjukoff's.

Experimental.

Paramucin Used as a Material.

Trembling orange jelly, weighing 2120 gm., was drawn from a large ovarian cyst with clinical diagnosis of cystoadenoma ovarii pseudomucinosum multiloculais sinistrum, and strained through a gauze under gentle suction. The filtrate (1880 gm.) was subjected to the preparation technique of Mitjukoff by the use of dilute HCl and HCl-containing alcohol and 85 gm. of paramucin in white powder were yielded. It was insoluble in water, alcohol and ether, but dissolved slowly in dilute NaOH (0.01 n. solution) and concentrated HCl.

Qualitative tests: Biuret, Molisch, Osaki-Turumi (both the indirect and direct) and Hiyama[4] reactions were distinctly positive. Tests for

hexuronic acid (Neuberg-Saneyoshi), pentose (aniline acetate test) and methylpentose (Rosenthaler; Widtsoe-Tollens) were negative. Hydrolysable sulfur and phosphorus were absent or traces, if present. The substance reduced hot Fehling solution faintly.

Quantitative figures are listed in Table I.

TABLE I.

Composition of the Preparation of Paramucin.

Analysis	Percentage	
Nitrogen*	10.1†	10.6‡
Hexosamine	15.5	
Non-amino sugar as galactose		
Indole r.	10.18//	
Orcinol r.	10.13//	10.09¶
α-Naphthol r. I §	10.84//	10.16¶
Mean	10.38	10.13
Acetyl	7.06**	
Ash	0.31	

* Micro Kjeldahl method. The obtained values resemble the corresponding Mitjukoff's value (10.54%). † The substance was preliminarily hydrolyzed with 1 n. H_2SO_4 in a sealed tube (100°C, 1½ h.). Sediments appeared thereby. Analysis was made with an aliquot part of the well-mixed suspension. ‡ A solution of the substance in 0.05 n. NaOH was used for the analysis. § Dische method[5]. // The remaining portion of the hydrolysate used for nitrogen determination (†) was decolourized by means of charcoal for this analysis. ¶ The solution in 0.05 n. NaOH above (‡) was diluted to subject to the colorimetric technique. ** Mitjukoff found her paramucin to involve 1.45% HCl. When this HCl content in the mucin as hydrochloride is taken into account, the real acetyl value is calculated at 5.35%.

Separation of the Amino Sugars in the Form of Hydrochloride.

The method applied to the glycidamins of chondro- and osseo-mucoid, which involve glucosamine, was used. But in the present case chondrosamine was obtained from the mother liquids of glucosamine after removal of remaining amino acids. The details will be given on one example.

10 gm. of the preparation of paramucin above were powdered and boiled under reflux with 100 cc. of 20% HCl containing 3.8 gm. of stannous chloride, after preliminary dissolution on a water-bath. The solution soon changed dark brown and a quite large amount of humin appeared. The hydrolysis was continued for 14 hours. The mixture was then diluted with an equal volume of water and tin precipitated with H_2S-gas, filtered off together with the dark sediments on a Büchner funnel and washed with boiling water thoroughly. The filtrate and washings were combined, condensed under reduced pressure to about 10 cc. and placed in an ice chest overnight. Crystals[6] appearing thereby were discarded

after separating on a funnel with suction and washing with some 20% HCl, and the filtrate and washing were united and evaporated by vacuum distillation as far as possible, and again distilled with several volumes of water to a syrup, which was transferred to a Dakin apparatus with a little water. Butanol was passed continuously for 10½ hours. To the residue were added 10 cc. of water, the insoluble part filtered off and the filtrate concentrated as above to about 0.5 cc. This was diluted with 1 cc. of 20% HCl and 7.5 cc. of methanol, and acetone added to faint cloudiness, whereupon crystallization began in a short while. Additional acetone was added cautiously from time to time (Addition of acetone was ceased when oil began to appear) and the deposit was collected on a suction funnel, washed twice with a few cc. of absolute alcohol and once with ether and dried in a desiccator ($CaCl_2$)—First fraction of glucosamine hydrochloride. Crop, 225 mgm.

The crude product was taken up in a small quantity of water (about 2 cc.) and after removing the insoluble part, a few drops of concentrated HCl and several volumes of methanol were added. Following addition of acetone similar to above caused crystallization. Yield, 200 mgm. To recrystallize once more, the substance was dissolved in water (0.5 cc.) again and a small portion of absolute alcohol added besides several drops of concentrated HCl. Without the aid of acetone crystallization (in plates) took place and progressed quickly. After one day, the deposit was brought onto a suction funnel, washed with absolute alcohol and ether and dried in a desiccator.

The product began charring at about 200°C. Though heated till 250°, it did not melt. Intensive hexosamine reaction (Elson and Morgan[7]) was shown.

N (micro Kjeldahl).
3.07 mgm. substance, 1.436 cc. 0.01 n. acid neutralized.
$C_6H_{13}O_5N \cdot HCl$. Calc., 6.50%; found, 6.55%.
NH_2–N (micro Van Slyke).
6.14 mgm. substance, 0.750 cc. N_2 (753.5 mm. Hg, 29°C)
$C_6H_{11}O_5NH_2 \cdot HCl$. Calc., 6.50%; found, 6.57%.
Optical rotation (in water). It showed mutarotation as is seen from Table II.

Judging from its extrapolated initial $[\alpha]_D$ of +91°, the product was a mixture of α- and β-glucosamine hydrochloride (the former predominating).

From the butanol extract above, when stood long, came out crystals of glucosamine hydrochloride. Hence after one day in an ice chest, the deposit was separated, washed and dried likewise as First fraction. This part amounted to 193 mgm.—Second fraction of glucosamine hydrochloride.

In the next the mother liquid of the first fraction of glucosamine hydro-

TABLE II.

Optical Rotation in Water of Glucosamine Hydrochloride from Paramucin. (Temperature, 27°C)

Time after dissolution min.	α deg.	$[\alpha]_D^{27} = \frac{\alpha \times 2.0388}{0.0461 \times 1.0023 \times l}$ deg.
5	+1.320	+86.8
30	+1.201	+79.0
60	+1.122	+73.8
60×2	+1.105	+72.7
60×4	+1.103	+72.5
60×24	+1.105	+72.7

chloride was distilled under diminished pressure and the residue re-evaporated several times with water. The final residue was dissolved in 5 cc. of water and an excess of 25% mercuric acetate solution added. The precipitates that appeared were filtered off. The filtrate and washings of the precipitates (water used) were combined and bubbled with H_2S, HgS filtered off and again the filtrate, after addition of a few drops of concentrated HCl, evaporated by vacuum distillation. The syrupy condensate was diluted with 4 cc. of absolute alcohol, removed from some insoluble substance and made up to about 10 cc. with methanol. Addition of acetone and petroleum ether produced only an oily deposit, which however changed on standing into a crystalline mass consisting mostly of prismatic thick needles and partly of thin needles. The both precipitants were supplemented in portions slowly and when a new precipitate showed no tendency to crystallize, the whole deposit was collected on a funnel, washed with a mixture of absolute alcohol and petroleum ether (1 : 1) and ether and dried. 255 mgm. were obtained. Recrystallization was carried out in like manner twice, but the use of petroleum ether was restricted to the smallest possible quantity in order to avoid the appearance of thin needle crystals. The yield of the product here was 190 mgm. It melted at 181°C under gas evolution and the mixed melting point test with β-chondrosamine hydrochloride from chondroitinsulfuric acid (F. P., 184°C (decomp.)) showed no depression (the mixture melted at 182°C (decomp.)).

N (micro Kjeldahl).
2.56 mgm. substance, 1.176 cc. 0.01 n. acid neutralized.
$C_6H_{13}NO_5 \cdot HCl$. Calc., 6.50%; found, 6.43%.
NH_2-N (micro Van Slyke).
5.13 mgm. substance, 0.590 cc. N_2 (763.8 mm. Hg, 18°C)
$C_6H_{11}O_5NH_2 \cdot HCl$. Calc., 6.50%; found, 6.45%.
Optical rotation in water.

The optical behaviour is illustrated in Table III.

TABLE III.

Optical Rotation in Water of Chondrosamine Hydrochloride from Paramucin.

Time after dissolution min.	α deg.	$[\alpha]_D^{17}=\frac{\alpha\times 2.0373}{0.041\times 1.0071\times 1}$ deg.
5	+1.030	+50.8
15	+1.180	+58.2
30	+1.320	+65.1
60	+1.502	+74.1
60×2	+1.710	+84.4
60×4	+1.908	+94.1
60×6	+1.930	+95.1
60×24	+1.936	+95.5

The figures in the table demonstrate that the product was β-chondrosamine hydrochloride. The extrapolated initial rotation was +45° and the equilibrium rotation +95.5°. These rotations agree with the respective corresponding values of Levene.[8]

Schiff's Bases of the Amino Sugars from Paramucin.

Identity of one of the separated amino sugars with glucosamine and of the other with chondrosamine was further established as derivatives with aldehydes.

The hexosamine hydrochloride less soluble in organic solvents.

2-Hydroxynaphthylidene glucosamine. 100 mgm. of the present amino sugar (hydrochloride) were taken for condensation with 2-hydroxynaphthaldehyde according to Jolles and Morgan.[9] The purified crystals were largely yellow thin needles and partly plates (Cf. Hiyama[10]). Yield, 75 mgm. F. P. 201°C (decomp.). The mixed melting point test showed no depression.

N (micro Kjeldahl method combined with reduction by means of HI and red phosphorus[11]).

7.06 mgm. substance, 2.073 cc. 0.01 n. acid neutralized.

$C_{17}H_{19}O_6N$. Calc., 4.20%; found, 4.11%.

The product had, at the 13th minute after dissolution, the rotation (in methanol) $[\alpha]_D^{25}=\frac{0.688\times 100}{0.35\times 1}=+196.6°$, which decreased quickly to

arrive at equilibrium in 4 hours. The value obtained after 24 hours from dissolution was $[\alpha]_D^{25}=\frac{0.614\times 100}{0.35\times 1}=+175.4°$ in accord with the equilibrium value of the corresponding Schiff's base of glucosamine (+174.4°; cf. Hiyama[10]). Thus the optical measurement disclosed that the α-isomer dominated in the product as in the corresponding derivative of glucosamine from chondromucoid-glycidamin.[10]

p-Methoxybenzylidene glucosamine. The procedure of Bergmann and Zervas[12] was followed. 90 mgm. of the amino sugar hydrochloride were taken up in 0.5 cc. of ice-cooled 1 n. NaOH solution and soon shaken with 0.5 cc. of anisaldehyde. Soon a crystalline (non-coloured prismatic needles) porridge formed. After 3½ hours in an ice-chest, it was brought on a suction funnel, washed with water, ice-cooled alcohol and ether in sequence and dried in a desiccator. Yield, 97 mgm. F. P. 165–166°C. (The substance prepared with glucosamine hydrochloride from chitin melted at the same temperature.) The mixed melting point test exhibited no depression. For analysis it was dried in vacuo (P_2O_5) at 80°C.

N (Friedrich method[11]).
5.630 mgm. substance, 1.860 cc. 0.01 n. acid neutralized.
$C_{14}H_{19}O_6N$. Calc., 4.71%; found, 4.62%.

The amino sugar more soluble in organic solvents.

2-Hydroxynaphthylidene chondrosamine. 100 mgm. of this amino sugar-fraction were subjected to the operations similar to above, and 72 mgm. of purified product (bright yellow needles) were obtained. It melted at 174°C (decomp.). The melting point of its mixture with the corresponding derivative of chondrosamine from chondroitinsulfuric acid (F. P. 175°C) (cf. Hiyama[13]) was the same.

N (Friedrich method)
6.12 mgm. substance, 1.844 cc. of 0.01 n. acid neutralized.
$C_{17}H_{19}O_6N$. Calc., 4.20%; found, 4.22%.
Optical rotation in methanol.
At the 11th minute after dissolution:
$[\alpha]_D^{16}=\frac{0.800\times 100}{0.383\times 1}=+208.9°$.
At equilibrium:
$[\alpha]_D^{16}=\frac{0.760\times 100}{0.383\times 1}=+198.4°$[14].

The hexosamine hydrochloride started with had the initial rotation $[\alpha]_D^{4}=+61.1°$ and hence was largely the β-isomer. It is noticeable that its Schiff's base took here α-form, as is indicated by the initial rotation, because, previously,[13] the Schiff's base in this form was produced from

α-chondrosamine and that in the β-form from β-chondrosamine by the same technique.

Identification of the Non-amino Sugar Component.

The hydrolysate of the glycidamin was fractioned and the non-amino sugar separated as methylphenylhydrazone as was processed by Sasaki[15] in an investigation of tendomucoid α, namely:—

5 gm. of the paramucin were taken up in 150 cc. of 1 n. H_2SO_4 and heated on a boiling water-bath for 5 hours under reflux. The substance got coloured pink at first and then dissolved slowly. To the dark brown hydrolysate was added, under cooling, just an enough quantity of the saturated $Ba(OH)_2$ in small portions. The precipitates were centrifuged and washed with 50 cc. of water three times. The supernatant red brown liquid and washings were combined and after removing the floating particles by filtration, distilled in vacuo to about 150 cc. (pH of the condensate, 4.4), then 100 cc. of 25% mercuric acetate solution were added. The precipitates (few)were rejected by centrifugation, and the pH of the supernatant liquid was moved with the saturated $Ba(OH)_2$ to 7.0, whereupon marked precipitation occurred. The brownish yellow deposit was centrifuged and followingly pressed on a suction funnel. From the supernatant liquid united with the filtrate barium was thrown down by means of 1 n. H_2SO_4 and through the centrifuged supernatant liquid mixed together with the washings of the centrifugate (600 cc. in total; pH, 3.4) H_2S-gas was passed. Since, however, centrifugation gave only a turbid supernatant liquid, this was acidified to a pH of about 2.0 with 1 n. H_2SO_4 (about 10 cc. used) and the saturated $Ba(OH)_2$ solution added in a quantity a little less than sufficient to remove all H_2SO_4. By centrifugation a clear liquid (700 cc.; pH, 2.6) was obtained this time, which was distilled under reduced pressure to about 100 cc. and H_2SO_4 precipitated completely with an equivalent quantity of $Ba(OH)_2$. The filtrate was exhausted witn ether (Ether was passed continuously for 8 hours; pH of the solution was raised to 4.2) and evaporated to 20 cc. at a low temperature (the pH lowered to 2.2).

8 cc. of the fluid were mixed with 0.4 cc. of methylphenylhydrazine and absolute alcohol added as much as to obtain a homogeneous solution. The mixture was heated for 10 minutes on a boiling water-bath and stood in a dark place. After several hours, the crystals, that had appeared, were collected on a funnel with suction, washed with 10 cc. of absolute alcohol and with ether and dried in a vacuum desiccator (P_2O_5). Yield, 140 mgm. Purification was carried out twice from hot 97% alcohol. The product (plates with silver lustre) melted at 190°C and the mixed melting point

test with galactose-methylphenylhydrazone (F. P. 190–191°C) showed no depression.

N (Friedrich method).

3.752 mgm. substance, 2.604 cc. 0.01 n. acid neutralized.

$C_6H_{12}O_5NN(CH_3)C_6H_5$. Calc., 9.85%; found, 9.73%.

To 4 cc. of the remaining fluid was added 0.2 cc. of freshly distilled phenylhydrazine and 0.5 cc. of 25% acetic acid and the mixture stood in an ice chamber. But no crystallization took place, suggesting the absence of mannose.

Thanks are due to the Education Department for a grant given through the Grant Committee for Scientific Researches in aid to us and also to Prof. T. Shinoda for the material in this work.

H. Masamune.

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7) Elson and Morgan, Bioch. J., 1933, **27**, 1824.

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Studies on the Influence of the Extrinsic Nervous System on the Intestinal Action Currents.

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Since Alvarez[1][2] first drew attention to the intestinal action currents, this problem has been studied further by Puestow,[3] Berkson[4] and other authorities in America. But in Japan, so far as I know, only Okuda and Maeda[5][6] published their reports on the problem in 1925.

In our laboratory, in 1943 Ito succeeded in obtaining the action currents from the bowel of a dog, and thereafter using rabbits chiefly, we made researches of the intestinal action currents which were influenced by drugs and which changed in pathologic situation.

Now I have studied the relation of extrinsic nervous system to the intestinal action currents and I have made experiments of the effects of several drugs upon the action currents from an excised piece of intestine, using rabbits to inquire into the action currents of the gut from which the influences of the extrinsic nerves were absolutely excluded, namely which was disconnected with the central nervous system.

A) The influence of the extrinsic nervous system.

Apparatus and experimental technique:

The abdomen of animals, which were kept from food for about 15 hours before the experiments and which 25 per cent. urethan anaesthetized, was opened and the lower ileum was exposed. Unipolar electrographic records were obtained by applying the zinc-zinc-sulfate moist thread electrodes attached to a string galvanometer. One of electrodes was attached to the serosal surface of the bowel and the other to the subcutaneous tissue, which had no potential change. The sensitivity of the galvanometer is 2.1×10^{-8} A. and periods of 4 seconds.

Results of experiments:

1) *Effects of spinal anaesthesia.*

5 rabbits were used. The upper part of the animal was fixed lying on its back and the lower sideways. The abdomen was opened and at first the electric records of the normal stage of the intestine were taken. Next, the changes of electric waves after the injection of 2 per cent. novocain-solution 0.5 cc. pro kilogram of the body weights were observed. A needle was inserted between the second and the third lumbar vertebrae after the removing of their transverse process and soon after the injection, the caudal part of the body was raised up and the inclination of about 30° was made. Iwashima[7] stated that by means of this method, the splanchnic nerves, which control the small intestine, were completely paralysed.

The electric waves in all cases did not change in their period, but grew larger in their amplitude obviously in a few minutes after the injection and this condition continued about an hour (Fig. 1).

Fig. 1

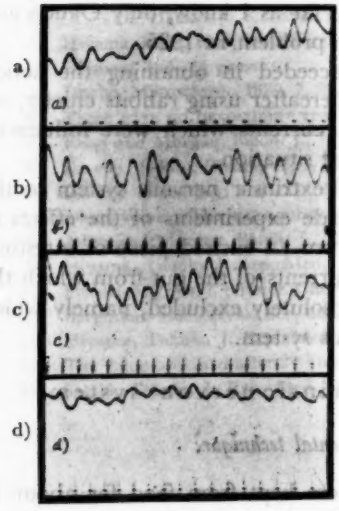

Fig. 2

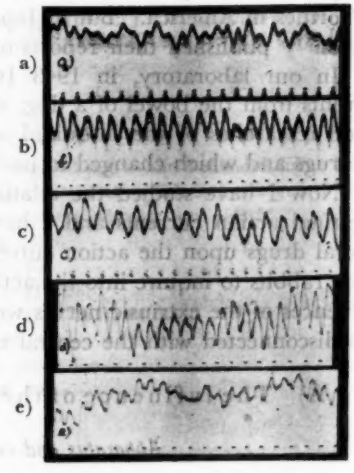

Fig. 1. The effect of the spinal anaesthesia; a) Before the injection. b) 5 minutes after the injection, large amplitude is seen. c) An hour after, that condition continues. d) One hundred minutes later, returned to normal. The time interval 5 seconds.

Fig. 2. The effect of cutting off the splanchnic nerves; a) Before the cutting. b) 40 minutes after the cutting. c) An hour later. d) 18 hours later. After the cutting, large amplitude is seen. e) 20 hours later, that condition returned to normal. The time interval 5 seconds.

2) *Effects of cutting off the splanchnic nerves.*

5 rabbits were used. The animal was fixed sideways and the abdomen was opened by upper midline incision. The normal records were obtained at first and next the both side splanchnic nerves were cut off extraperitoneally and thereafter the changes of the electric waves were observed.

The waves became regular and grew larger in their amplitude, but did not change in their period. This condition continued several hours. In one case, this change was noted to return to the normal condition after about 20 hours (Fig. 2).

3) *Effects of cutting off the vagusnerve.*

5 rabbits were used. The abdomen was opened with upper midline incision, the normal electric waves were obtained and then the change of the electric waves after cutting off the both side vagi right below diaphragma were observed.

The electric waves became larger in their period and smaller in their amplitude, but in one case the waves enlarged obviously, namely the results were indefinite (Fig. 3).

Fig. 3

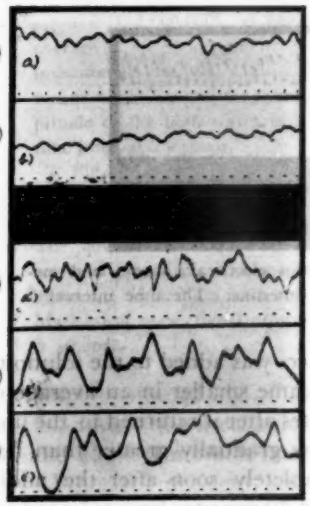

Fig. 4

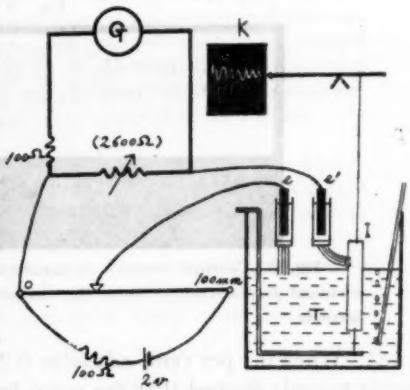

Fig. 3. The effect of cutting off the vagal nerves; a) Before the cutting. b) 40 minutes after the cutting, amplitude became smaller and period larger. In the other case: a') Before the cutting. b') 25 minutes after the cutting, both amplitude and period became larger. c') 40 minutes after, that condition continued. The time interval 5 seconds.

Fig. 4. Circuit of the apparatus. G: Galvanometer. K: Kymographion. I: Intestine. T: Tyrode's solution. e, e': Electrodes.

B) The effects of several drugs upon the excised intestine.

Apparatus and experimental technique:

A piece of intestine about 3 cm. long was excised from the lower ileum of a rabbit. It was allowed to contract rhythmically in Tyrode's solution 200 cc. kept at about 38°C. Namely by Magnus'[8] method the mechanical records were made on a soot-paper and simultaneously the electric ones were made on a bromide paper by unipolar method, in which one electrode was attached to the serosal surface of the intestine and the other to the surface of the solution as shown in Figure 4. I used the same galvanometer as in the experiments on an intact intestine.

Results of experiments:

1) *Normal records.* I observed the more regular wave than in the case of an intact intestine. Its period corresponded to the mechanical wave clearly, but its amplitude, namely the potential change, did not always coincide with the intensity of intestinal activity (Fig. 5).

Fig. 5

Fig. 5. Normal waves. A simultaneous electrical (above) and mechanical record (below) from the excised intestine. The time interval 5 seconds.

2) When 0.1 per cent. *adrenaline* 0.2 cc. was added to the solution, the electric records showed that the wave became smaller in an average of its amplitude and period, and about 10 minutes after it returned to the normal condition and then 20 minutes after grew gradually greater than before. The mechanical movement stopped completely soon after the addition, about 10 minutes later began to move and 30 minutes later enlarged rather than before (Fig. 6).

3) Immediately after 1 per cent. *atropine* 0.5 cc. was added to the solution, I observed that the electric wave became a very irregular one, that is, the small waves were piled up over. About 5 minutes later a special wave appeared: one electric wave corresponds to two mechanical ones.

Fig. 6

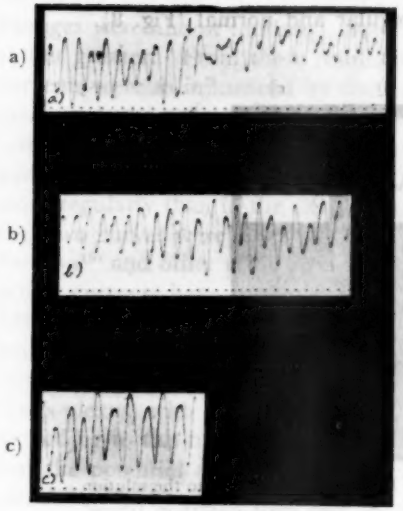

Fig. 7

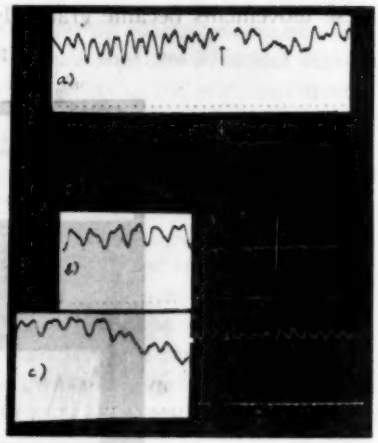

Fig. 6. The effect of adrenaline; Adrenaline was added to Tyrode's solution (1: 1,000,000). a) The electric wave (above) became small only in an average of its period and amplitude, while the movement (below) stopped immediately after the addition. b) 12 minutes after, the electric wave returned to normal and the intestine began to move. c) 30 minutes after, amplitude of the both waves enlarged rather than before the addition. The time interval 5 seconds.

Fig. 7. The effect of atropine; Atropine was added to the solution (1: 40,000). a) Electrical record (above) soon after the addition showed that the waves were very irregular, in which small ones were piled up over. The movement (below) became very weak. b) 5 minutes after, one electric wave corresponded to two mechanical ones. The mechanical wave began to move. c) 35 minutes later, special waves (left) above mentioned continued. Mechanical ones (right) returned gradually to normal. The time interval 5 seconds.

This change continued about 30 minutes. The intestinal movement were clearly inhibited: the mechanical wave almost disappeared or became very weak, and about 5 minutes later it returned gradually to normal (Fig. 7).

4) When 0.1 per cent. *pilocarpine* 0.5 cc. was added to the solution, I could observe that very great oscillation occurred soon after the addition, a few minutes later the electric waves became larger in their amplitude and relatively more regular. And then about 30 minutes later, they returned to normal.

The mechanical records showed that the tetanic-like contraction occurred soon after the addition, after a few minutes very vigorous and

irregular movements began and continued about 30 minutes. Thereafter these movements became gradually regular and normal (Fig. 8).

Fig. 8

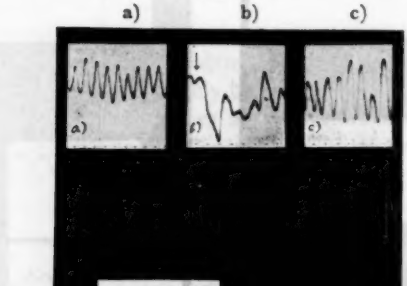

Fig. 8. The effect of pilocarpine; Pilocarpine was added to the solution (1:400,000). a) Before the addition. b) Very great oscillation (above) occurred soon after the addition. The mechanical movement (below) showed the tetanic-like contraction. c) 10 minutes after, the electric waves became larger in amplitude and relatively regular (above). Very vigorous and irregular movements began (below). d) 30 minutes after, both records returned gradually to normal. The time interval 5 seconds.

Comment.

It is generally recognized that effects of the splanchnic nerves are inhibitory on intestinal movements and if these nerves are paralysed, the intestinal movements are excited. And it is ascribed to the paralysis of the splanchnicus that the intestinal movements increase, when spinal anaesthesia is performed. And it is quite well known that when the vagal nerves are stimulated, the intestinal movements increase, but recently Kure[9] and his fellow-workers have suggested that the vagal nerves have something to do only with the stomach and the upper part of the small intestine and the greater part of it is innerved by the spinal parasympathicus. Their investigations have aroused general interest. Namely the influences of the vagus on the lower part of the small intestine are indefinite. In any case, it is recognized that the splanchnicus and the vagus give each other the opposite effects on the intestinal movements.

In my experiments, I have observed that the intestinal action currents either by the section of the splanchnicus or by spinal anaesthesia showed the identical changes, in which the electric waves increased in their regularity and grew greater in their amplitude. On the other hand, when

the vagus was cut off, the electrical records showed that the electrical changes were almost inhibited, but in one case the electric waves became rather greater. From these results it was clear that the intestinal action currents were so influenced by the extrinsic nerves as the intestinal movements. And in the experiments, using the excised intestine which was completely separated from the central nervous system, I found the electrical and the mechanical curves changed almost parallel with each other and more regularly than in the case of intact intestine, but I also found the fact that two curves were dissociated by adding certain drugs, as described by Berkson[10] and other physicians. So it may be assumed that the intestinal action currents have intimate relations to the movements but the former differ from the latter in their origin. In other words, the obtained electrogram may be the one that was made by combination of the action currents from nervous cell itself of the local nervous system (Auerbach's and Meiszner's plexus) with the action currents following the intestinal movements. According to this hypothesis, it may be attributed to the myenteric plexus that the potential changes are able to occur without the intestinal movements.

Conclusion.

1. After spinal anaesthesia and in the case of cutting off the splanchnicus, the electric waves increased their regularity and grew larger in their amplitude, and they were inhibited in most cases of cutting off the vagus, except in one case of the experiments.

2. When I obtained the electrical records from the excised intestine of rabbits, which was disconnected with the central nervous system, it was found that the electric waves became more regular than in the case of the intact intestine, which was controled through the extrinsic nerves by the central nervous system. Therefore I think that the essential intestinal action currents show the rhythmical impulse occurring from the local nervous system and having nothing to do with the extrinsic nerves.

3. I investigated, using the excised intestine, the effects of some drugs on the intestinal action currents and its movements. The electrical and mechanical curve changed almost parallel with each other, but it was found that two curves were dissociated when the certain drugs were applied.

4. Hence it was assumed that the extrinsic nervous system had some effects on the intestinal action currents, but Auerbach's and Meiszner's plexus played a leading rôle of the occurrence of the intestinal action currents.

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Experimental Studies on the Action Currents of the Small Intestine in the Case of Acute Intestinal Obstruction and Peritonitis.

By

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(Received for publication, November 5, 1948)

The action currents of small intestine have been studied only by Alvarez & Mahoney and a few other investigators. But there are no detailed studies on the intestine in pathologic situations yet, for instance, such as acute obstruction or peritonitis. So I have investigated the action currents of small intestine and its motility in the case of experimental acute intestinal obstruction and peritonitis to see the changes of them and especially the differences between these two situations.

Experimental Technique:

Using healthy rabbits, acute mechanical intestinal obstruction was made by a simple ligature placed in their terminal ileum, and acute peritonitis was caused by the method, in which the fresh fecal emulsion dissolved in saline solution about 15 ccm. was injected in the peritoneal cavities or by cutting the top of appendix. The abdomen was opened again after settled time, the requested part of the guts was exposed and the potential changes were photographed on bromide paper by the unipolar method, in which one electrode was attached to the surface of the guts and the other to the subcutaneous tissue. The circuit of this apparatus was shown in Fig. 1.

Fig. 1

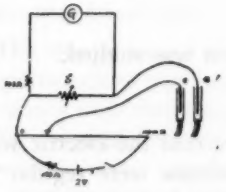

Fig. 1. Circuit of the apparatus. G.: Galvanometer. e, e': Electrodes. S: Shunt, Resistance of 2600 Ω

A string galvanometer, whose sensitivity was 2.1×10^{-8} A. was used, and the electrode was zinc-zinc-sulfate moist thread electrode. The mechanical records of the intestinal activity were separately made on a soot-paper by the modified Trendelenburg's method soon

after obtaining the electric records. (Fig. 2.)

Fig. 2

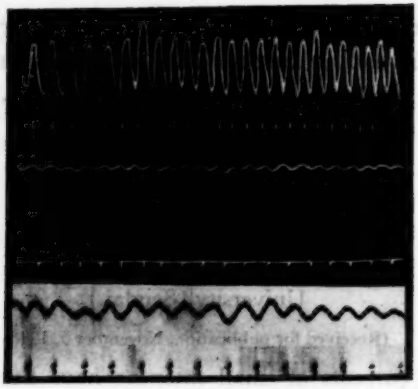

Fig. 2. Normal mechanical and electrical records from lower ileum of a rabbit. a), b) normal mechanical waves on different condition. c) normal electric waves. The time interval 5 minutes.

Result of Experiments:

I. Acute obstruction.

13 rabbits were used. At the early stage of acute obstruction, which was occurred by my method, the intestine above the site of obstruction distended slightly and its wall was hyperaemic. The dilatation of the wall gradually increased, and became thin due to the congestion of intestinal contents, which were coloured brownish black and seen through the wall. At the last stage the wall became violet red due to hyperaemia of high degree. The electric and the mechanical records were obtained from two sites: the one is just above obstruction and the other about 100 cm. oral from the former.

A. At first the change after obstruction was studied.

a) About 2 or 3 hours after obstruction.

It was already investigated in our laboratory that the electric waves and the mechanical ones from normal small intestine were regular and rhythmical in their form, period and amplitude, but in 2 or 3 hours after obstruction it was found at the part just above obstruction and the upper part of the closed loops that the electric waves became irregular and enlarged in their period and amplitude, while the intestinal movements were irregular and inhibited. (Fig. 3. a).

Fig. 3. (a, b, c, d)

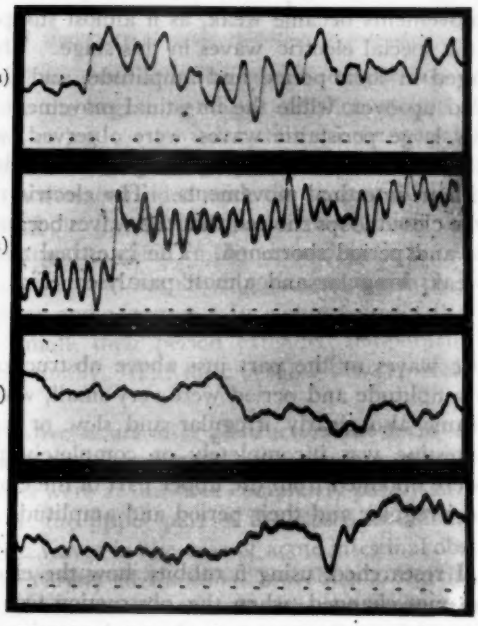

Fig. 3. Variations of the electric waves during acute obstruction. Records obtained from the point just above obstruction. a) Two and half hours after obstruction. Irregular and large waves were shown. At this time the movements were also irregular and slow. b) Four hours after. The waves became relatively regular and rapid. At this time the contractions were vigorous. c) Twenty seven hours after. The waves became very irregular and their amplitude and period shortened, while the contraction almost invisible. d) Fourty five hours after. Irregular and small electric waves still continued. This time the intestine became incompletely or completely paralysed.

b) 4 to 6 hours after.

The electric waves at the part just above obstruction became regular, their period shortened and the amplitude rather enlarged, and sometimes large waves were recognized. At this time, the intestinal movements were relatively regular and vigorous, but occasionally I could see the paralysis of the movements. At the upper part of the closed loops there were no changes of the electric waves, and its movements stayed almost normal too (Fig. 3. b).

c) 18 to 30 hours after.

The electric waves at the part just above obstruction gradually be-

came irregular again, their amplitude small and period shortened, while the intestinal movements became weak, as if almost stopped. But sometimes I found the special electric waves in this stage. These waves were ones that enlarged in their period and amplitude, and the normal small waves were piled up over, while the intestinal movement almost stopped and occasionally large peristaltic waves were observed, which coincided with the special electric waves and it was recognized that there were potential changes without intestinal movements. The electric records from the upper part of the closed loops showed that the waves became irregular, and their amplitude and period shortened. The intestinal movements at this part became weak, irregular and almost paralysed (Fig. 3. c).

d) About 45 hours after.

The electric waves at the part just above obstruction became very irregular, their amplitude and period were very small, while the intestinal movements became also clearly irregular and slow or invisible, namely this part of intestine was incompletely or completely paralysed. The waves, which were obtained from the upper part of the closed loops in this stage, were also irregular and their period and amplitude shortened (Fig. 3. d).

B. Next, I researched, using 5 rabbits, how the electric waves and the mechanical ones changed, when the obstruction was removed. The observation was made at the part just above obstruction. In one group of experiments I removed it about 4 hours after, when both the electric and the mechanical waves were much excited. About 15 minutes after removing, the electric waves became irregular, their amplitude shortened, and period enlarged, and they returned gradually to normal, sixty minutes later, while the mechanical ones in these cases became slow and also recovered normal about an hour later.

In another group, obstruction was removed about 20 hours after, and the intestine was imcompletely paralysed. About twenty minutes after removing it, the electric waves became more irregular than before, their amplitude enlarged and the period extended obviously. The intestinal movements were relatively regular and slow. The observation about sixty minutes after the removing showed that the electric waves tended to become normal and regular gradually. The movements returned also to be rapid and normal. The special electric waves, so-called the piled waves, almost disappeared this time. In one case, in which the normal electric waves did not return by removing obstruction, the large and slow waves appeared, as seen in the last stage of acute peritonitis. The animal in this experiment died shortly after the investigation. So it is suspected that the appearance of such large and slow electric waves as this indicates the worse

prognosis of the diseases (Fig. 7).

Summary of acute intestinal obstruction.

The intestinal movements in the case of acute obstruction are inhibited almost moveless in the beginning stage, and then excited and at last completely paralysed owing to the exhaustion of the muscle. The electric records obtained from the point just above obstruction showed that the changes of these waves coincided with the variety of the intestinal movements. Namely about 2 or 3 hours after obstruction the waves became irregular and enlarged in their amplitude and period. 4 to 6 hours after their period shortened and amplitude enlarged regularly, while the movements were regular and vigorous. In eighteen to thirty hours their amplitude became small, their period extended temporarily and thereafter they shortened gradually. The form of these waves increased in irregularity.

About fourty-five hours after obstruction the form of waves became very irregular and their amplitude and period smaller, namely the intestinal movements were at this time completely paralysed. These changes occurred slowly at the upper part of the closed intestine. As mentioned above, the electric waves in the case of acute intestinal obstruction change parallel with the variety of intestinal movements, but it was recognized that the electrical changes continued without intestinal motility.

After the removing obstruction the intestinal movements were temporarily inhibited and returned to normal gradually, while the electric records obtained from the part just above obstruction showed that the waves became more irregular than before and then tended to be regular and normal.

II. Acute Peritonitis.

Now there are numerous methods experimentally to produce peritonitis in animals, but I was successful in producing it by the injection of the fecal emulsion in the peritoneal cavities of 13 rabbits. There was only a small amount of peritoneal fluid and light hyperaemia at the serosa of intestine in the primary stage of the peritonitis, but as the disease became more advanced, the fluid was bloody and purulent, its quantity increased and the hyperaemia became intense.

The electric records were obtained from the lower ileum in the inflammatory stage as these. The intestinal movements were almost or completely paralysed. The electric waves became gradually small and irregular. In one case I recognized the piled waves, which were found in a certain stage of acute obstruction, and in other case, I observed a series of very small regular and rapid waves. Such special waves as these re-

turned to normal immediately, when acetylcholine and vagostigmin were applied and in about thirty or fourty minutes after the application these waves became again abnormal; namely their amplitude and period were small and irregular. Moreover I could obtain the electric waves from the bowel of a rabbit on the verge of death due to peritonitis (Fig. 4. 5. 6).

Fig. 4

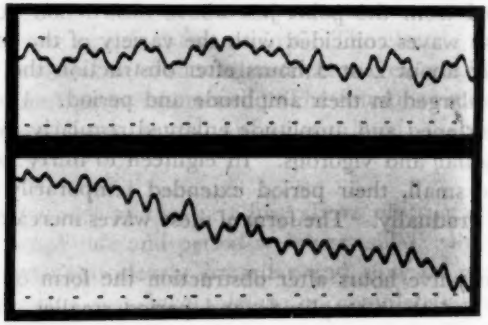

Fig. 4. Peritonitis. Ten hours after the injection of fecal emulsion. The electric wave grew smaller.

Fig. 5

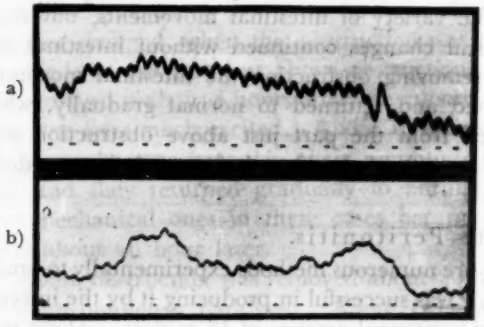

Fig. 5. Peritonitis of high degree. a) The very small, rapid and relatively regular waves continued. The mechanical movements were almost invisible. b) Occasionally the large and irregular waves occurred.

In this case the intestinal movement almost stopped, while the irregular and slow potential changes were able to be photographed (Fig. 7).

Summary of acute peritonitis.

There have been many discussions about the etiology of the intestinal paralyse due to acute peritonitis. Notnagel and other authors attributed its cause to the excitement of the splanchnical nerves owing to bacterial

Fig. 6

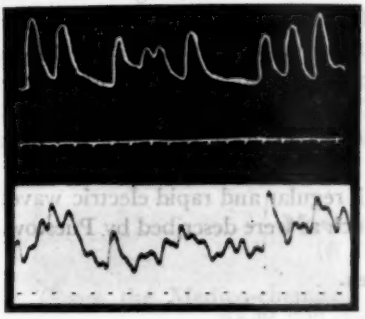

Fig. 7

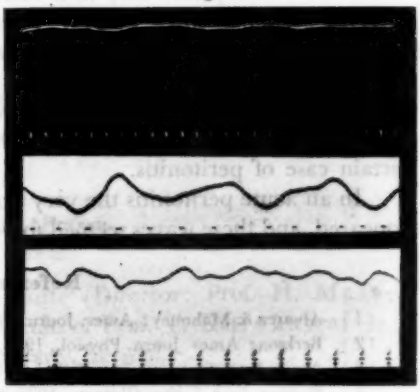

Fig. 6. The special waves, so-called the piled waves in the case of peritonitis. The electrical record (below) was obtained soon after obtaining the mechanical ones (above).

Fig. 7. The electrogram from the bowel of a rabbit on the verge of death due to peritonitis. The mechanical record (above) showed that the contraction almost invisible, but the electrogram (below) showed that the slow potential changes still existed.

toxin. Budde attached importance to Bac. Welchii, and acidosis occuring in blood or Histamin is also now looked upon as a cause of intestinal paralyse. In any case, when acute peritonitis occurs, the intestinal movements become weaker and there are occasionally vigorous peristaltic waves, but in the last stage there is no visible movement, that is to say, complete paralysis of the movement occurs. I obtained electrogram in the experimental peritonitis. My records showed that the electric waves became smaller and rapid, as Puestow described, but I could not see the disappearance of the electrical responses, until the animal died.

III. Comparison of the electrogram in the case of acute intestinal obstruction and acute peritonitis.

In the early stage of acute obstruction (in 4 to 6 hours after obstruction), I recognized the appearance of regular, great and rapid potential changes, which I could not find in the case of acute peritonitis.

Through all the courses of acute peritonitis, generally I found the small waves and occasionally very rapid and small oscillations, which did not appear in the case of acute obstruction.

Conclusion.

Using rabbits, I experimentally caused acute mechanical intestinal

obstruction and peritonitis, and researched into the changes of action currents of the diseased bowel. The electric waves in the case of acute obstruction almost changed parallel with the variation of intestinal movements and in the early stage the very regular, great and rapid waves appeared. And I found the special waves, which were large and over which the normal waves were piled up. These waves were obtained, too in a certain case of peritonitis.

In an acute peritonitis the very small, regular and rapid electric waves appeared, and these waves seemed to be such as were described by Puestow.

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A Toxic Substance in Carcinomatous Ascitic Fluid.

First Report.

By

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There have been several poisonous substances informed of in cancer. Of old, Kullmann[1] prepared a hemolytic extract from mammary and uterine cancers by extraction with a glycerol-saline solution. The active substance was coctostable and soluble in water as well as in alcohol. Grafe and Römer[2] separated from gastric juice of patients with gastric carcinoma a lipoid containing oleic acid in the molecule. This also possessed hemolytic potency which was adapted by the authors to cancer diagnosis. Recently, Iwatsuru and others[3] from Kozawa's clinic pointed to the appearance of a heat-stable and undialyzable poison (KIK-factor) in the secretion of cancer stomachs. It is reported that, on intravenous injection to rabbits of the gastric juice containing this factor, reduction of the erythrocyte number and hemoglobin content of blood occurs. Almost simultaneously, Sakai[4] in Onodera's clinic isolated from the urine of cancer patients crystals, which were insoluble in water and soluble in alcohol, acetone and ether, and caused, when subcutaneously applied to rats, the proliferation of spleen. The compound was unhydrolyzable and resembled cholesterol.

Bard[5] observed rapid dissolution of erythrocytes in hemorrhagic carcinomatous exsudates, but this was not confirmed by Well.[6]

The present writer separated four poisonous fractions from a cancerous ascitic fluid. Two of them were obtained from the precipitate in the bag when dialysed, and were intensively biuret-positive and non-electrodialyzable. The others, one biuret-negative and the other biuret-positive, were the ones that migrate into the anode compartment on electrodialysis. These killed a mouse by intraperitoneal injection of 5 mgm. for 10 gm. bodyweight, whereas those were less toxic. If the toxicity of the fractions is ascribable to a single compound, it must be an acid of a small molecular

weight, because the part in the non-electrodialyzable fractions is regarded to have been retained inside the membrane through complex formation with proteins. It could not be taken up in plain, acid and alkaline alcohol from the fractions, and the farctions did not give the Osaki-Turumi (for hexosamine) and Pettenkofer (for bile acid) reactions. Isolation of the active factor in a pure state was not accomplished due to shortage of material, although several attempts were allowed.

Experimental.

Fractionation of the ascitic fluid was tried as below. Toxicity of the fractions thereby obtained was determined by intraperitoneal injection of them to a mouse. For this purpose a weighed amount of the fractions was dissolved or suspended in 1 cc. of physiological saline and roughly neutralized. The term "toxic," which will appear below, signifies that the animal was killed within 24 hours.

A viscous slightly hemorrhagic exsudate was drawn from the carcinomatous peritoneal cavity of a severely cachectic patient with diagnosis (clinical and patho-anatomical) of gastric cancer and liver metastasis, and blood cells centrifuged off. 100 cc. of the supernatant yellow fluid were condensed to 23 cc., by transferring in drops through a glass capillary into a vacuum distillation flask. The appearing precipitate was rejected by centrifugation and ether was passed exhaustively through the alkline (pH, 8.4) thick fluid (Fr. A) at first as such and then after acidulation (to pH 4.8) with acetic acid. In the meantime again a precipitate came out. The ether extracts were combined and evaporated. The yellowish remainder, insoluble in water, amounted to 170 mgm.—Fr. B. The precipitate above was separated (by centrifugation) from the fluid, washed with water and dried—Fr. C (430 mgm., coloured brownisch) and the fluid and the washings of the precipitate were united and dialyzed (Thymol was used as a preservative) in a beaker against changes of distilled water through cellophane, until alkalization of renewed outer liquid no longer occurred. The dialysates were evaporated together to dryness (Fr. D_o, 700 mgm.). The content of the bag was centrifuged and the centrifugate and the residue of the supernatant fluid after distillation (under reduced pressure) dried in a desiccator (P_2O_5). They weighed 550 mgm. (Fr. D_{ip}) and 2.25 gm. (Fr. D_{il}) respectively. Both were tinged brown.

Frs. C, D_o, D_{il} and D_{ip} all killed a mouse in a dose of 20 mgm. per 10 gm. bodyweight within 24 hours,[7] while Fr. B did not in the same dose.[8] The original exsudate freed from blood cells was not proved toxic even in a dose of 1 cc. per gm. bodyweight. This suggested the existence in it of some counteracting substance, but, putting aside this problem, the writer

undertook to separate the toxic factor from D's. The smallest fraction C was not further treated.

1. D_o. 680 mgm. of D_o were taken up in 38 cc. of water[9] and the centrifuged yellowish supernatant liquid (pH 9.2) was condensed and after drying up, washed with many 10 cc. portions of 98% alcohol, until no more colouring matter came out. The insoluble part and the evaporated remainder of the washings were dried in a desiccator.

The obtained substances amounted to 350 and 240 mgm. respectively. The former (Fr. D_{oan}, white powder) killed a mouse, when 10 mgm. of it were injected for 10 gm. bodyweight, while, even in double the dose the latter did not. The toxic factor of the alcohol-insoluble fraction migrated into the anode compartment on electrodialysis, which was carried out in a Pauli apparatus (collodion membrane, 200 volts) by applying all of D_{oan} as suspended[10] in 100 cc. of water (pH of the solution was >9.0) and continued until the pH of the solution arrived at neutral, by changing from time to time the water in the outer compartments. After vacuum distillation of the fluids to dryness, it was found that about 1/3 (Fr. E_o') of the substance had moved to the anode and 2/3 (Fr. E_o'''') to the cathode, only a trace (E_o'') remaining in the middle compartment. The minimum lethal dose of E_o'[11], which was biuret-negative, was 5 mgm. for 10 gm. bodyweight but E_o'''' was non-toxic.

2. Three 100 mgm. portions of D_{il} were taken and extracted with absolute alcohol repeatedly, one immediately, a second after kneading with 1 drop of water and adding 0.15 cc. 1 n. HCl to pH 2.0, and a third after kneading with 3 drops of water and adding 0.1 n. NaOH (0.27 cc.) to pH 9.0. The volume of alcohol used at first in each case was 20 cc. The alcohol extracts were evaporated to dryness[12] under reduced pressure, the acid and the alkaline having been neutralized. The alcohol insoluble rests[13] were dried in a desiccator. These amounted to 94, 50 and 80 mgm. respectively and nearly as toxic as D_{il} itself, while the parts dissolved in alcohol[12] were not.

On the other hand, a 600 mgm. portion of the same fraction (D_{il}) was subjected, after taking up in 30 cc. water and removing the small undissolved part, to electrodialysis similarly as the suspension of D_{oan} (s. above). Thereby 34.5 mgm. migrated to the anode (E_{il}') and 79.5 mgm. to the cathode (E_{il}''''). In the middle compartment, 254 mgm. remained in solution (E_{il}'') and 19.5 mgm. precipitated (E_{il}'''). Of E_{il}'—E_{il}'''', only E_{il}'[14] was toxic (M.L.D., 5 mgm.). This gave no Pettenkofer reaction for bile acid, but did give Molisch and biuret reactions.

3. D_{ip}. 320 mgm. of this fraction were suspended in 28 cc. of water and electrodialyzed as above. However, here no toxicity was found not

only in the part at the positive electode but also in that at the negative electrode. From the solution in the middle compartment were recovered 94.5 mgm. substance (E_{ip}'') and the sediments in here weighed 174 mgm. (E_{ip}'''), when dry. These substances killed a mouse in a dose of 10 and 20 mgm. respectively for 10 gm. bodyweight. 154 mgm. of E_{ip}''' were then dissolved in water by the aid of alkali (NaOH added to pH 9.0) and precipitated with acid (acetic acid added to pH 5.4). The precipitate (74 mgm. after drying) had a minimum lethal dose of 10 mgm. per 10 gm. bodyweight of mouse. It was biuret and Molisch-positive, but did not give the positive Osaki-Turumi reactions (the direct and indirect). The mother liquid exhibited no poisonousness. It was also tried to further purify E_{ip}'' by means of glacial acetic acid, but toxicity was recognized neither in the glacial acetic acid-insoluble part nor in the precipitate from the extract by ether.

Description of purification trials that were unsuccessful is omitted.

The costs of this work were defrayed by a grant from the Grant Committee for Scientific Researches of the Education Department, which is gratefully acknowledged. H. Masamune.

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7) The minimum lethal dose of D_{ip} was 10 mgm. per 10 gm. bodyweight.

8) Injected as a suspension in physiological saline.

9) The insoluble part amounted to 26 mgm.

10) About 2/3 of the substance was found to dissolve in water on neutralization. In electrodialysis all of the substance went into solution.

11) Its solution in physiological saline was acid (pH, 4.4) and neutralization produced distinct precipitation. Toxicity was examined by injecting the neutral suspension.

12) The part, which was insoluble in alcohol at alkaline reaction, was also insoluble in water. The others, alcohol-soluble and -insoluble, dissolved in plain water.

13) The amounts of the parts which dissolved in alcohol were so small that they were injected per 10 gm. bodyweight in the doses, which corresponded to 30 mgm. of the substance before alcohol treatment.

14) This was not perfectly soluble in physiological saline. The suspension obtained was acid and therefore neutralized for injection.

The Tohoku Journal of Experimental Medicine, Vol. 51, Nos. 3 & 4, 1949.

Do Cancerous Ascitic Fluids Contain Mucoitin ?*

By

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(Received for publication, November 5, 1948)

Meyer and Chaffee[1] isolated mucoitin (so-called hyaluronic acid) from the viscid pleural fluid of a patient with malignat tumors of the pleura and peritonium diagnosed as mesothelioma or endothelioma. According to the authors, the viscous factor of the fluid was this polysaccharide acid and had originated in tumor cells. They further imagined that the viscous carbohydrate, which Kabat[2] had obtained from fowl sarcoma and its cyst, is similar, if not identical, to mucoitin. It must be of significance to inquire as to whether or not mucoitin exists in the carcinomatous exsudate in serous cavities, because tumor cells here arise from the embryonal origin belonging to a different category.

Our material was a carcinomatous ascitic fluid from a patient with stomach cancer. It was submitted at first to dialysis and then to electrodialysis. After electrodialysis the insoluble part and the liquid in the middle compartment were separated. And the former was subdivided into a fraction insoluble in dilute alkali, that soluble in dilute alkali but insoluble in dilute acid and that soluble in dilute alkali as well as in acid. The latter was condensed and precipitated with alcohol. From each of these fractions a part was taken and treated with glacial acetic acid to remove phosphorus-containing substances. However, all the residues undissolved gave the negative naphthoresorcinol test for hexuronic acid, and were revealed by analysis to be simple proteins with comparatively small carbohydrate content. Thus it was proved that mucoitin was absent in the exsudate. To the same conclusion we came from the result of an attempt to separate mucoitin, if present, by means of formaldehyde. For this purpose were used fractions before treatment with glacial acetic acid and one of the final products above.

* 123rd report of Masamune and co-workers' Biochemical Studies on Carbohydrates.

Experimental.

Fractionation of the Ascitic Fluid.

870 cc. of a fairly viscid exsudate (pH 7.6) were drawn from the peritoneal cavity of a patient with diagnosis: carcinoma ventriculi with peritonitis carcinomatosa. The fluid was dialysed through cellophane against running water for 3 days and the content of the bag (the liquid (pH, 7.0) together with the voluminous precipitates, which had appeared) was subjected to electrodialysis (Water in the outer compartments was changed many times, until the pH of renewed portions was not shifted), and centrifuged. The centrifugate was washed with water, alcohol and ether, and dried in vacuo (Fr. P, 8.8 gm.). The supernatant liquid and water used for washing the centrifugate were combined and evaporated under reduced pressure, and the ropy condensate (transparent) dehydrated with alcohol, washed with alcohol and ether and dried (Fr. L, 6.1 gm.).

In the next, Fr. P was kneaded with some water, water increased to 180 cc. and dilute alkali (NaOH) dropped in cautiously to constant pH of 9.0. The viscous solution was separated from the insoluble part (Fr. P_1; this amounted to 0.25 gm. when dried) and acidified to pH 5.6 with dilute HCl, whereby precipitation occurred. The deposit was centrifuged, washed and dried as above (Fr. P_2; 4.129 gm.), and the mother liquid evaporated to syrupy consistency and precipitated with alcohol and washed and dried similar to above (Fr. P_3; 1.2 gm.). Since these fractions contained phosphorus (ammonium molybdate test), they were further treated with glacial acetic acid as follows. Substance was kneaded with a little water, taking the whole P_1 and 0.6 and 0.5 gm. of P_2 and P_3 respectively, diluted with drop by drop of glacial acetic acid at first and then glacial acetic acid further added as much as to precipitate all the part insoluble in it. The precipitate was washed with alcohol and ether and dried. On repeating this process twice, fractions free from phosphorus were obtained—Fr. P_1', 180 mgm.; Fr. P_2', 390 mgm.; Fr. P_3', 280 mgm. 0.6 gm. of L was also liberated likewise from phosphorus—Fr. L', 270 mgm.

Those products were all insoluble in water and, among them, P_1' and P_2' were insoluble also in dilute alkali and acid. P_3' was soluble in dilute alkali and mostly soluble in acid at pH 2.0, but precipitation occurred on lowering the pH of the acid solution. L' dissolved in alkali but did not in acid. All resembled one another qualitatively: negative ammonium molybdate (for phosphorus), aniline acetate (for pentose and hexuronic acid) and naphthoresorcinol (for hexuronic acid) tests; positive biuret, Molisch and Liebermann (for tryptophane) tests. Quantitative analysis gave the figures in Table I.

TABLE I.

Composition of the Non-electrodialyzable and Glacial·Acetic Acid-insoluble Fractions of a Carcinomatous Ascitic Fluid.

(Figures express percentages.)

Fraction \ Analysis	N	Hexosamine	Sugar† as glucose by orcinol reaction	Ash
P_1'*	11.6	1.74	2.74	5.03
P_2'*	13.0	3.60	5.30	1.37
P_3'*	12.9	6.05	5.38	1.59
L'*	13.1	0.51	1.43	0.41

* Means the products in the text.

† Substance was hydrolysed for 3 hours with 1 n. H_2SO_4 in an ampoule (temp., 100°C) and the filtrate analysed. The colours developed by orcinol, diphenylamine and indole did not coincide in intensity, as expressed as any of hexoses (glucose, mannose and galactose).

A Search for Mucoitin by means of Formaldehyde.[3]

1.5 gm. of Fr. P_2, 0.8 gm. of Fr. P_3, 3.0 gm. of Fr. L and 0.05 gm. of Fr. P_1', which were left unused in the experiment above (in total, 5.35 gm.), were suspended together in 100 cc. of water and alkalized with 1% Na_2CO_3 to pH 8.4, whereby almost all went into solution. After addition of 10 cc. of formalin and bringing back the lowered pH to 7.8, the mixture was made up to the volume of 200 cc. with water. Then it was shaken on a machine for 24 hours (pH at this stage, 7.6) and centrifuged. The supernatant liquid and the washings of the centrifugate combined were acidified to pH 4.6 (30% acetic acid used). The voluminous precipitate, that came out, was centrifuged and washed with two 20 cc. portions of dilute acetic acid of the same pH and the supernatant liquid and the washings were united and precipitated with 4 volumes of 95% alcohol and anhydrous sodium acetate. This precipitate was washed with alcohol repeatedly, with addition of anhydrous sodium acetate when necessary, and dried in a desiccator. 250 mgm. of brownish substance were obtained.

The product above was extracted exhaustively with water. The extracts (some were slightly turbid) were evaporated together under diminished pressure to about 8 cc., and 40 cc. of absolute alcohol and 0.2 gm. of anhydrous sodium acetate added. The precipitate was separated, washed with alcohol and ether and dried. It amounted to 180 mgm. On re-extractions of it and evaporation and precipitation of the extracts, the amount of substance decreased to 50 mgm. Its naphthoresorcinol test of Neuberg and Saneyoshi for hexuronic acid resulted in negative. Biuret

and Molisch reactions were given and phosphorus was detected.

To remove phosphorus the substance was treated with glacial acetic acid. Namely, it was kneaded with 2 drops of water, glacial acetic acid added at first in drops and afterwards in larger portions (in total, 15 cc.) and finally anhydrous sodium acetate (0.3 gm.) added to complete the precipitation. The centrifugate was washed with alcohol many times and with ether twice and dried. This procedure with glacial acetic acid and anhydrous sodium acetate was effected twice more, using here ether together because of insufficient precipitation by the former agents alone. Yield, 27 mgm. This phosphorus-free substance also gave neither Goldschmiedt reaction with α-naphthol nor Neuberg-Saneyoshi test with naphthoresorcinol nor aniline acetate reaction. Biuret and Molisch reactions were positive.

To note in passing, the product at the end was a mixture of a water-insoluble, a water-soluble but acid(pH, 3.8)-insoluble and a water- and acid-soluble substance, the last of which was biuret- and Molisch-positive and gave the direct reaction of Osaki and Turumi but not the indirect.

We thank to the Grant Committee for Scientific Researches of the Education Department for a grant in aid to us. H. Masamune.

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On the Effects of Several Drugs upon the Action Currents of the Small Intestine.

By

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(Received for publication, November 7, 1948)

The action currents of the small intestine were first obtained by Alvarez[1], and they have been studied further by his assistants, Puestow[2] and Castelton.[3] But this research remains still in an early stage. I report here the action currents of rabbits intestine and chiefly the effects of several drugs upon them. A dog was used also as an experimental animal.

Apparatus and Experimental Technique.

The string galvanometer, whose sensitivity was 2.1×10^{-8} amp., and a period of four seconds, was used. The circuit was planned by Motokawa, in which the light from a reflecting mirror is always on the slit of camera, which is called the compensating circuit.

In this circuit the resistance of shunts was 1,000 ohm in the case of a dog, but when a rabbit, the intestine of which has weak currents, was used, the most adequate resistance was 2,600 ohm. The distance between camera and galvanometer is about 150 cm. and the deflection of about 5 cm. corresponds to 1 mv. The camera covered with dark dram is Zimmermann's Kymographion rolled by bromide paper. The electrode is zinc-zinc sulfate moist thread electrode.

We have now various investigation-methods of the intestinal activity to be compared with this curve of the potential change, but in this work the modified method of Trendelenburg's was used; it is Yadagai's method.[4]

The abdomen of the with urethane anaesthetized rabbit was opened, the requested part of intestine (chiefly upper ileum) was fixed to the apparatus and one of electrodes was applied to the surface of the intestine, the other being attached to the surface of the wound or the subcutaneous tissue, which had no potential changes; it is named unipolar lead. In this way the electrical records were taken. At the same time or separately, the mechanical records were made by straw lever on the soot papers.

Results of Experiments.

I. Normal character of the action currents.

At first, the relation between the wave of potential changes and that of the mechanical ones has been researched.

The movement of intestine exposed from abdominal cavity is, as well known, at first vigorous, but gradually grows weaker and weaker and at last almost motionless. The electrical records from intestine could be always obtained, even when it seemed motionless. Of course, when the movements continue strongly the potential changes appear with the same rhythm as that of the intestinal activity, and the contractionsphase corresponds electrically to the negative and the relaxations to the positive.

The rate and the amplitude: The small intestine has the same constitution all over from duodenum to ileum, but the activity at the different parts of gut differ from each other, as described by Alvarez[5] and he stated that the potential changes of duodenum are rapid and small, and ileum presents greater and more regular waves than the former. In this research eight rabbits were used and the electrical records were made from following five portions; duodenum, upper jejunum, lower jejunum, upper ileum and lower ileum, and their differences were shown on Fig. 1. At duodenum there are about eighteen times a minute of potential changes and at the lower ileum about ten times a minute and their amplitude becomes greater at caudal portion than at oral. Moreover, three dogs were used and the records were taken respectively, that is, from the lower ileum and from the portion about 100 cm. oral from there, and it was also observed that there occur slower and greater waves at caudal part than at oral.

Fig. 1

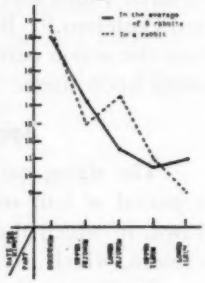

Fig. 1. Rate of potential change in different part of the small intestine.

Form of the electrical wave obtained: Berkson,[6] who used string galvanometer, stated he had got the characteristic form of the electrical wave from the small intestine of a rabbit by means of the unipolar method, and if by means of the bipolar, an inversion of wave occurred, which was similar to the wave described by Puestow, who used the apparatus of electrocardiogram and took out the electrogram of dog's intestine.

In my experiments only a simple wave of sinus-curve could be obtained by string galvanometer. This looked like the wave early shown by Alvarez. Besides, the electrogram has been taken from the intestine of rabbits and dogs by the improved apparatus of electrocardiogram, which has four tubes, but I could not yet conclude, in a case of rabbits, whether there is or

not the typical definite form of the electrical wave having the quick oscillations, as described by Puestow. (Fig. 2)

Fig. 2

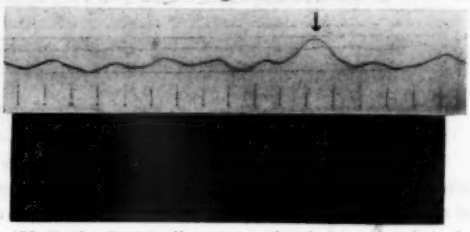

Fig. 2. Normal waves at ileum: A simultaneous electrical (above) and mechanical record (below) from intact intestine of rabbit. The time interval two seconds. ↓ indicates the movement of body.

Interpretation of the electrical records: There are two methodes, by which we understand now the curve of action currents.

In one, for instance, in electrocardiogram, we pay special attentions to the definite form of the wave. In the case of electroencephalogram, the variation of its period and amplitude are important. On the curve of the intestinal action currents, in my work, an importance was attached to its period and amplitude, because the typical form of wave which Berkson and others speak of does not always appear as in E.K.G., but on special condition. When the variations of the action currents according to the portions were studied, the average rate and amplitude of all waves were examined, that occurred during the observation for about five minutes and when the effects of drugs upon them were researched the two factors were examined one or two minutes before and after the applications of drugs.

II. Inquiry of the effects of drugs.

Next, it was inquired what changes the electrical wave has in intact intestine of rabbits, when its activity changes by the application of various drugs. The electrical records and the mechanical ones were all made separately, but at the same conditions.

a) *Nicotine*: 0.1 percent. Per Kg. of body weight 0.5 ccm.. Into the ear vein injected.

The electrical records were obtained from eleven cases of rabbits and the mechanical ones from two cases. While the mechanical wave grew immediately greater and after about ninety minutes became most vigorous, the electrical wave was largest in period and amplitude nearly ten minutes after the application of nicotine, and most regular, and in seven cases out of eleven, later became smaller, its period being 1.2–1.5 seconds. (Fig. 3)

b) *Pilocarpine*: 1 percent. Per Kg. of body we ght 0.2 ccm. Intraven-

Fig. 3

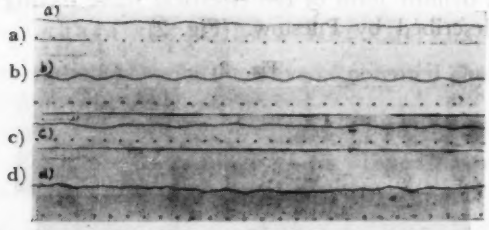

Fig. 3. The effect of nicotine: Electrical record. Nicotine (1.2 ccm. 1:1000) injected into ear vein. a) Normal rhythm. b) Six minutes later the vigorous and regular waves are seen. c) Thirty minutes after the application they become small and rapid. d) Sixty minutes after the small and rapid waves continue. The time interval two seconds.

ously injected.

The electrical records were obtained from four cases of rabbits and the mechanical ones from two cases. Immediately after the injection it was observed even by naked eyes that the mechanical wave was made greater and on the other side the electrical wave was greater both in period and amplitude by the application of this drug. (Fig. 4)

Fig. 4

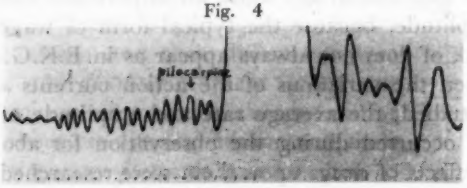

Fig. 4. The effect of pilocarpine: Electrical record. Pilocarpine (0.6 ccm. 1:100) injected into ear vein. Immediately erratic oscillation set up. A short time later the large waves occur, and the mechanical movement is vigorous even by naked eye. The time interval five second.

c) *Vagostigmine*: 0.5 percent. Per Kg. of body weight 0.5 ccm. Subcutaneously injected.

The electrical records were obtained from three cases of rabbits and the mechanical ones from a rabbit. When this drug was injected, the mechanical wave was made greater five minutes after the application and the influence lasted for about thirty minutes, and the electrical wave became slightly greater in its period and amplitude on an average a few minutes after the injection.

d) *Acetylcholine*: Per Kg. of body weight 0.05 mg. Intravenously injected.

The electrical records were obtained from three cases of rabbits and the mechanical ones from two cases. While the mechanical wave became immediately greater, the electrical wave grew greater soon after the application.

e) *Histamine*: 0.05 percent. Per Kg. of body weight 0.2 ccm. Intravenously injected.

The electrical records were obtained from two cases of rabbits and the mechanical ones from two cases. While the mechanical wave was inhibitted soon after the injection and then gradually became greater, the electrical one was scarcely influenced soon after the injection, but about three minutes later the period and amplitude were greater than before.

f) *Atropine*: 1 percent. Per Kg. of body weight 0.2 ccm. Intravenously injected.

The electrical records were obtained from three cases of rabbits and the mechanical ones from a rabbit. While the mechanical wave immediately disappeared, the electrical one continued not influenced.

g) *Adrenaline*: 0.1 percent. Per Kg. of body weight 0.2 ccm. Intravenously injected.

The electrical records were obtained from three cases of rabbits and the mechanical ones from two cases of the same rabbits. Immediately the mechanical wave stopped, and about six minutes later it began to move and then grew gradually greater than before. The electrical records showed that the wave was small in period and amplitude soon after the application and about thirty minutes later the greater wave was obtained. (Fig. 5)

Fig. 5

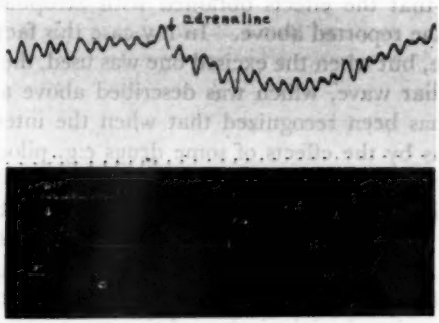

Fig. 5. The effect of adrenaline: Adrenaline (0.5 ccm. 1:1000) injected into ear vein. The movement stops immediately (below) but in the same condition the electrical waves become small and rapid in an average. The time interval five seconds.

Moreover, effects of adrenaline, atropine and pilocarpine on the excised segment of rabbit's intestine were studied.

A piece of the excised strip of intestine about 3 cm. long was allowed to contract rhythmically in Tyrode's solution 200 ccm. kept at about 38°C. One electrode was attached to the serosal surface of an intestinal strip, and the other to the surface of fluid. The electrical records were made by means of the method mentioned above. At the same time the mechanical records were made on a soot paper.

a) *Adrenaline*: When 0.1 percent adrenaline 0.2 ccm. was added to the solution, the obtained effects were similar to those in the case of an intact intestine.

b) *Atropine*: When 1 percent atropine 0.2 ccm. was added, the mechanical movement was immediately inhibitted, while the electrical wave continued, which had a special form; one electrical wave corresponds to two mechanical waves.

c) *Pilocarpine*: When 1 percent pilocarpine 0.2 ccm. was added, the same effects were taken as in the case of an intact intestine.

Summary.

Berkson[7] and others observed that when nicotine was applied, the intestinal activity became vigorous, but its action currents disappeared. In my experiments the disappearance of action currents could not be recognized, but the electrical records showed the changes with very small waves a few minutes after the application. And they described that when epinephrine was used the activity of intestine stopped at once, but the electrical wave was hardly influenced. In my experiments it was influenced clearly in both cases of an intact intestine and excised one. They stated moreover that the effects obtained with atropine were similar to those by adrenaline reported above. In my case this fact was observed in an intact intestine, but when the excised one was used, the electrical records showed the peculiar wave, which was described above already.

Further, it has been recognized that when the intestinal movements are more vigorous by the effects of some drugs e.g. pilocarpine, vagostigmin, acetylcholine or histamine, the electrical wave becomes greater in its period and amplitude and when the movements are inhibitted, it becomes smaller. It was true in case of adrenaline, as was mentioned above.

Namely in my experiments it has been shown that two responses, which are potential changes and mechanical ones, react with some drugs differently, and at the physiological conditions or by means of some drugs the period and the amplitude of the electrical curve change generally in parallel with the variation of the intestinal activity. However, the degree of variations which two responses demonstrate differs from each other. So there is probably included in the electrical curve an important factor which may be distinguished from the action currents made by musclar activity. Accordingly it will be assumed that the intestinal action currents contain two elements; that is, the action currents which have their origin in the intrinsic nervous system or in the chemical changes in the muscle, and which are made by the intestinal activity.

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(5) Alvarez; Amer. J. Physiol., 1914, **35**, 177.
(6) Berkson; Amer. J. Physiol., 1932, **102**, 683.
(7) Berkson; Proc. Soc. Exp. Biolog. and Med., 1932, **30**, 205.

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Influences of the Ventricular Systole Upon the Cardiac Sinus Rhythm in Man.

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(Received for publication, November 27, 1949)

Roth and Kisch[1] reported of an interesting study concerning the inhibitory effects of the ventricular systole upon the rate of impulse formation in the sinus node of man. They attributed these effects to the reflexes evoked in the pressoreceptors in the vasosensory areas.

Recently we confirmed such effects in a patient exhibiting the interference dissociation due to the incomplete A–V block. A part of this study is reported here briefly.

The patient is a peasant, 48 years old. Chief complaints: shortness of breath and distress in chest, both aggravated on exercises. Pulse rate 34 per min. or so, apparently regular. ECG revealed the incomplete A–V block and the interference dissociation.

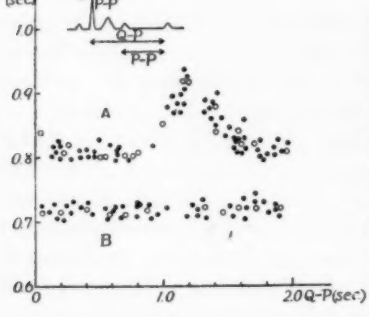

Fig. 1. Changes in sinus rhythm along the phases of time between two ventricular systoles, before (A) and after (B) atropinization. Data from PP cycles in succession are plotted as circles.

By taking long records of ECG we got a general relation between RP and PP intervals along all phases of the RR interval (points A in the Fig. 1). There is a definite tendency of slowing in sinus rhthm at a certain time after the preceding R. The longest PP cycles are not necessarily the next ones from the preceding R as pointed out by Roth and Kisch. The length of PP interval is a function of the time elapsed after the foregoing R. The slowing begins fairly suddenly at 0.8 sec. after R, attains its maximum at 1.2 sec. to decline rather slowly thereafter.

By atropinization such slowing effects disappeared completely (points B in Fig. 1).

Details of this study as well as the analysis of ECG will be reported elsewhere.

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A Group-specific Substance of Lipoid Nature in a Liver Metastasis of Gastric Cancer.

First Report.*

By

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It will be of importance to decide whether or not group-specific substances are present in malignant tumors, which take an immature embryonic type of tissue, because fetus in the earlier stages of development exhibit no group specifity as is known. Oh-Uti,[1] a colleague of mine, isolated from stomach cancers, carbohydrates and a glycidamin which were of a low grade of differentiation in the mentioned aspect. The present account deals with an active lipoid in this kind of malignant tumor. The material was a liver metastasis of gastric cancer. There were obtained fractions specifically inhibitory to the isoagglutination of B erythrocytes (the patient belonged to Group B). The activity of four fractions finally obtained was recognized at a dilution of 1: 800 in one of them and in the others at a dilution of 1: 1600. Early fractions inhibited the agglutination but non-specificaily, and the specifity became manifest in the later ones. This is comparable to the observations of Lattes et al[2] as well as of Eisler and Moritsch[3] on the extracts of erythrocytes. The final fractions above showed different solubilities, one being soluble in almost all organic solvents and insoluble in water and the others soluble only in a few organic solvents such as benzene, chloroform and hot tetralin but very readily in water. Each of them contained nitrogen and phosphorus and gave Thudicum's reaction for psychosin and Molisch's for sugar, all in accord with the findings by Hamasato[4] on the group lipoids from erythrocytes. Quantitative-analytically, however, they diverged from one another markedly. Their approximate equivalent ratios of sugar as galactose : phosphorus : nitrogen were found 1: 5: 10; ?: 1: 8; 1: 2: 9; 1: 1: 3. The preparation possessing the equivalent ratios at the end of the com-

* 1st report of Masamune and co-workers' Chemistry and Biology of Lipoids.

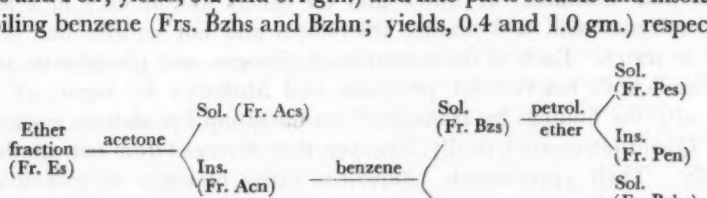
ponents was most water-soluble and contained the largest amount of sugar, viz., 17.5%. Inferences from the above results will be described in the paragraph of discussion. I examined also if lipoid fractions of the tumor give rise to hemolysis whose ditails will be given in the experimental part.

It ought to be added here that investigations of group lipoids from other sources than erythrocytes have never before been made.

Experimental.

Procedure of Fractionation and the Anti-agglutinative Potency of the Fractions.

A large matastasis in liver of gastric cancer (the person belonged to Group B), weighing 2200 gm., were cut into small pieces, washed with water to free from blood and masticated. The pieces were placed in 1.5 *l* of 95% alcohol for 10 days, and after drying, powdered in a mortar and fibers removed. Then 77.5 gm. of the substance corresponding to 583 gm. fresh tissue were extracted by boiling with alcohol repeatedly, until no hot alcohol-soluble substance came out. The extracts were evaporated together under reduced pressure, the appearing crystals being separated half way. The remaining syrup was dried up in a vacuum desiccator (H_2SO_4) and subjected to ether extraction in a Soxhlet apparatus. On the other hand, the residue after treatment with alcohol was also extracted similarly with ether. And these ether extracts were evaporated together in a shallow wide vessel on a water-bath by the aid of a fan. The brownish syrupy residue (Fr. Es; 18.3 gm.) was fractioned with 40 cc. portions (6 portions) of dry acetone. The acetone extracts were evaporated together similar to above, and the remaining yellow oil dried in vacuo. This (Fr. Acs) weighed 13.1 gm. The acetone-insoluble residue (brownish resinous substance—Fr. Acn) was divided into parts soluble and inscluble in benzene[5] (Frs. Bzs and Bzn; yeilds, 3.8 and 1.4 gm.) and the obtained fractions further into parts soluble and insoluble in cold petroleum ether (Frs. Pes and Pen; yields, 3.2 and 0.4 gm.) and into parts soluble and insoluble in boiling benzene (Frs. Bzhs and Bzhn; yields, 0.4 and 1.0 gm.) respectively.



(Solvents, whose temperature is not particularly mentioned, were cold.)

The anti-agglutinative power of the above fractions was examined by our usual method, by emulsifying Frs. Pen, Bzhs and Bzhn in physiological saline without and the others with the aid of alkali. The results are embodied in Table I, which shows that the fractions, the acetone-soluble fraction (Acs) excepted, inhibited the agglutination, though non-specifically. The residue after extraction of Es was almost devoid of this potency.

TABLE I.

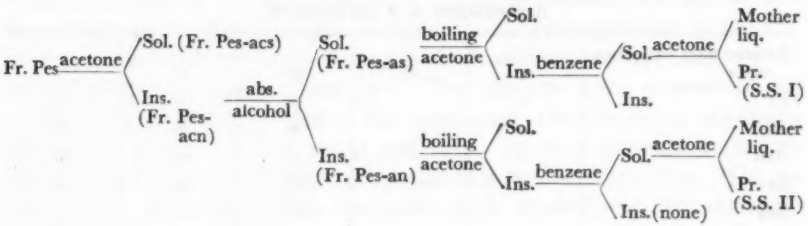
Agglutination of A and B Erythrocytes by Normal Antisera Treated with Early Fractions of the Tumor Lipoids.

A and B sera had titres 160 and 80 respectively.

Fraction	Dilution of substance				
	1% Original	×2	×5	×10	×10²
Agglutination of A Erythrocytes.					
Residue after extraction of Es	##	##	##	##	
Es	−		−	++	##
Acs	+		##	##	##
Acn	−		−	++	##
Bzs	−		−	+	++
Bzn	−		−	+	++
Pes	−		−	+	++
Pen	−		−	+	++
Bzhs	−		−	±	++
Bzhn	−	±	+	++	++
Agglutination of B Brythrocytes.					
Residue after extraction of Es	±	+	++	##	
Es	−		−	+	++
Acs	+		##	##	##
Acn	−		−	+	++
Bzs	−		−	+	++
Bzn	−		−	±	++
Pes	−		−	±	++
Pen	−		−	−	++
Bzhs	−		−	±	++
Bzhn	−	−	+	+	++

Subdivision of Fr. Pes. It was tried to liberate this fraction (3.2 gm.) completely from cold-acetone-soluble substances. For the purpose, ten 30 cc. changes of acetone were used, each time with subsequent drying. The

remaining very hygroscopic solid clump—Fr. Pes-acn—weighed 2.3 gm. 1 gm. of it (the remaining part was lost by mistake) was next fractioned with absolute alcohol (three 30 cc. portions), the alcohol extracts evaporated in vacuo and the rest dried after washing (twice) with acetone—Fr. Pes-as. This was treated with boiling acetone (one 30 cc. and one 10 cc. portion) and the insoluble part taken up in 5 cc. benzene (The insoluble part, which was a trace, was discarded.), precipitated with acetone(Nothing remained in the mother liquid.) and, after washing with acetone, dried—Group-specific Substance (S.S.) I (hygroscopic brownish powder); yield, 136.8 mgm. The amount of the substance, which had passed over into boiling acetone, was the resembling. The absolute alcohol-insoluble rest—Fr. Pes-an—was washed repeatedly with cold (six 20 cc. portions) and boiling (one 30 cc. and one 10 cc. portion) acetone and, after drying, precipitated from benzene[6] and dried again as was S.S.I—S.S. II (brown powder; 61 mgm.).



The anti-agglutinative activity was elevated and became specific (Table II). The acetone-soluble fractions were inactive.

Subdivision of Fr. Pen. 330 mgm. of this fraction were taken and at first divided into absolute alcohol-soluble (The extracts were evaporated together; the dried up residue (white powder) weighed 267 mgm.—Fr. Pen-as.) and -insoluble (brownish powder, 18 mgm.—Fr. Pen-an) parts (One 40 cc. and two 30 cc. portions of alcohol were used.). Both showed specific inhibition of B blood cells from isoagglutination (s. Table III), but due to shortage of material further fractionation of the latter was abandoned, and from the former 251 mgm. were taken and extracted with anhydrous benzene (three 40 cc. portions). Most of the substance did not dissolve. The insoluble fraction—Fr. Pen-bzn (yellowish solid)—amounted to 190 mgm. and the soluble—Fr. Pen-bzs (yellowish solid)—to 40 mgm. Fr. Pen-bzn was treated thoroughly with petroleum ether, acetone and ether. The amount of the remaining brownish powder was 119 mgm.—S.S. III.

The stages are schematized in next page, and the serological potencies embodied in Table III.

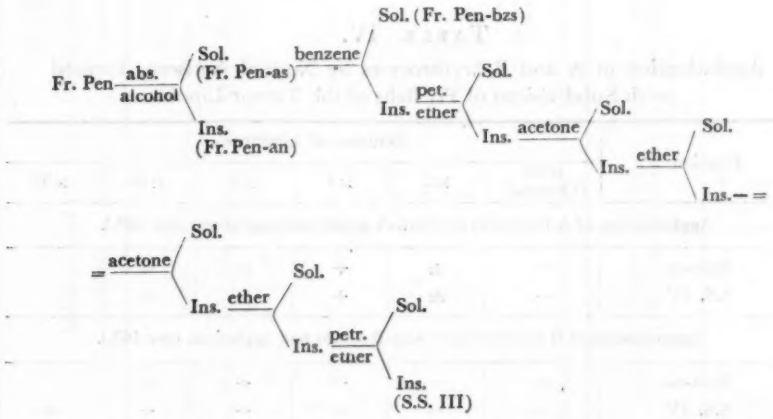
Subdivision of Fr. Bzhs. From the Bzhs fraction (0.4 gm.) were removed acetone-soluble substances (Five 20 cc. portions of acetone were

TABLE II.

Agglutination of A and B Erythrocytes by Normal Antisera, Treated with Subdivisions of Fr. Pes of the Tumor Lipoids.

The substances were taken up in physiological saline without alkali.

Fraction	Dilution of substance					
	1% (Original)	×2	×4	×8	×16	×32
Agglutination of A Erythrocytes (titre of anti-A serum, 80).						
Pes-acn		−	±	+	++	+++
Pes-as		−	±	+	++	+++
Pes-an		−	+	+	++	+++
S.S. I		−	+	++	+++	+++
S.S. II		−	+	++	+++	+++
Agglutination of B Erythrocytes (titre of anti-B serum, 160).						
Pes-acn		−	−	−	+	++
Pes-as	−	−	−	−	+	++
Pes-an	−	−	±	+	++	+++
S.S. I		−	−	−	−	+
S.S. II		−	−	−	+	++



used.). The residue contained only a trace of absolute alcohol-soluble substance—Fr. Bzhs-as. After washing with this solvent and drying, the residue—Fr. Bzhs-an—was boiled with a 30 cc. and a 10 cc. portion of acetone under reflux, and then precipitated from benzene with acetone as were S.S's I and II, and dried in a desiccator—S.S. IV (hygroscopic brownish powder, 43.1 mgm.). The fractions, which dissolved in acetone

TABLE III.

Agglutination of A and B Erythrocytes by Normal Antisera, Treated with Subdivisions of Fr. Pen of the Tumor Lipoids.

The substances were taken up in physiological saline without alkali.

Fraction	Dilution of substance									
	1% (Original)	×2	×4	×5	×8	×10	×16	×32	×64	×10²
Agglutination of A Erythrocytes (The employed B serum had agglutinin titre 160.).										
Pen-as	−			+		++				+++
Pen-an	−			+		+				++
Pen-bzs	−	−	±		+		++	+++	+++	
S.S. III	−	−	−		+		+	++	++	
Agglutination of B Erythrocytes (The employed A serum had agglutinin titre 160.).										
Pen-as	−			−		±				++
Pen-an	−			−		−				++
Pen-bzs	−	−	−		−		+	++	+++	
S.S. III	−	−	−		−		−	+	+	

(cold and hot), were not active. Table IV shows the potencies of the others.

TABLE IV.

Agglutination of A and B Erythrocytes by Normal Antisera, Treated with Subdivisions of Fr. Bzhs of the Tumor Lipoids.

Fraction	Dilution of substance					
	0.5% (Original)	×2	×4	×8	×16	×32
Agglutination of A Erythrocytes (Anti-A serum had agglutinin titre 160.).						
Bzhs-an	−	±	+	++	++	
S.S. IV	−	±	+	++	+++	
Agglutination of B Erythrocytes (Anti-B serum had agglutinin titre 160.).						
Bzhs-an	−	−	−	+	++	
S.S. IV	−	−	−	−	+	++

Fr. Bzhn. 100 mgm. of this were fractioned with absolute alcohol and the insoluble part (36.0 mgm.) furtheron with water (three 2 cc. portions). The substances taken up by alcohol (Fr. Bzhn-as) and those taken up by water (Fr. Bzhn-ws) were recovered by evaporation and drying in a desiccator: 62 and 22 mgm. were yielded respectively. Present subdivision,

Table V.

Solubility of the Final Group-specific Fractions of the Tumor Lipoids.

(– insoluble, ± faintly sol., + partly sol., ++ mostly sol., +++ completely sol.)

Fraction	Solvent																	
	Abs. alcohol		Ether		Petr. ether		Acetone		Benzene		Tetralin		Chloroform		Gl. acet. acid		Water	
	Cold	Hot	Cold	Hot	Cold	Hot	Cold	Hot	Cold	Hot	Cold	Hot	Cold	Hot	Cold	Hot	Cold	Hot
S.S. I	+++	+++	+++	+++	+++	+++	–	–	+++	+++	+++	+++	+++	+++			–	–
S.S. II	–	++	–	–	–	–	–	–	+++	+++	–	+++ (40–60°C)	+++	+++			++	+++
S.S. III	+	+++	–	–	–	–	–	–	++	+++	±	+++ (60°C)	++	++	–		++	+++
S.S. IV	–	–	–	–	–	–	–	–	+++	+++	–	+++ (40–60°C)	+++	+++			+++	+++

Table VI.

Sequels of Chemical Assays on the Final Group-specific Fractions of the Tumor Lipoids.

Fraction \ Test	Biuret	Sugar (Molisch)	Nitrogen (Lassaigne)	Phosphorus (ammonium molybdate test)	Sulfur (Lassaigne)	Psychosin (Thudicum)	Amino sugar (Hamasato & Akakura)	Methylpentose (Rosenthaler)	Pentose (Aniline acetate test)	Uronic acid (Neuberg & Saneyoshi)	Cholesterol (Liebermann & Burchard)
S.S. I	–	+	+	+	–	+	–	–	–	–	±
S.S. II	–	+	+	+	–	+	±	–	–		++
S.S. III	±	+	+	+	–	+	+	–	–		–
S.S. IV	–	+	+	+	–	++	+	–	–		–

however, did not raise the anti-agglutinative power nor gave rise to specific action.

Solubility of the Group-specific Products (S.S.'s I–IV).

Solubility of S.S.'s is illustrated in Table V.

S.S.I dissolved well in most usual organic solvents except acetone, and did not in water, although it formed an emulsion. However, the other group substances were soluble in water and in particular S.S.IV gave a complete solution in the cold. These were soluble in benzene and chloroform with ease, but with difficulty in general organic solvents. S.S.'s II and III dissolved in alcohol to different extents and IV did not at all. In short, the group-specific fractions exhibited inorganic character in solubility more and more from left to right in the order of I. III. II. and IV. The watery solutions of S.S.'s II–IV were not precipitated by addition of dilute HCl to pH 2.4 or less.

Qualitative and Quantitative Chemical Properties of the Group-specific Products (Tables VI and VII).

All of S.S.'s I–IV gave positive Molisch (for ordinary sugar) and Thudicum (for psychosin) reactions but negative tests for hexuronic acid (Neuberg and Saneyoshi), methylpentose (Rosenthaler), pentose (aniline

TABLE VII.

Composition of the Final Group-specific Fractions of the Tumor Lipoids.

	Sugar as galactose			Phoshorus*	Nitrogen†	Ash‡
	Indole r.	Orcinol r.	Diphenyl-amine r. I			
	In per cent					
S.S. I	3.77§	1.9§		3.36	3.05	11.0
S.S. II				1.07	3.63	9.08
S.S. III	7.49	6.95	7.49	2.56	5.07	7.47
S.S. IV	16.4	13.9	15.6	2.60	3.62	8.10
	In equivalents per equivalent weight					
S.S. I	1.0			5.2	10.4	
S.S. II				1.0	7.5	
S.S. III	1.0			2.0	8.7	
S.S. IV	1.0			0.93	2.9	

* Haneda, Sinokawa and Yasuoka's modification of Plimmer method.[7]
† Micro Kjeldahl method. ‡ Micro analysis.
§ The substance was analyzed as an eumlsion in water.

TABLE VIII.

Occurrence of Hemolysis by the Fractions of the Tumor Lipoids.

(– not hemolyzed, ± faintly hem., + partially hem., ++ half hem., +++ mostly hem., ++++ completely hem.)

Fraction	Dilution of substance										
	1:10^2	1: 5×10^2	1:10^3	1: 2×10^3	1: 4×10^3	1: 8×10^3	1: 1.6×10^4	1: 3.2×10^4	1: 6.4×10^4	1: 1.28×10^5	1: 2.56×10^5
S.S. I		–	–	–	–	–	–	–	–	–	–
S.S. II		–	–	–	–	–	–	–	–	–	–
S.S. III		+++	+++	+++	+++	+++	+	–	–	–	–
S.S. IV		+++	++	+	+	+	+	–	–	–	–
Bzhn	–	–	–	–	–	–	–	–	–	–	
Bzhn-as	+++	++	±	±	–	–	–	–	–	–	
Bzhn-ws	–	–	–	–	–	–	–	–	–	–	
Bzhs-as	+++	+++	+++	++	+	–	–	–	–	–	
Pen-an	++	±	–	–	–	–	–	–	–	–	
Acs	+++	+++	+++	++	±	–	–	–	–	–	
Residue after extraction of Es	–	–	–	–	–	–	–	–	–	–	

acetate test) and sulfur (Lassaigne). By the analytical method of amino sugar of Hamasato and Akakura, like colouration took place in III and IV as in hexosamine-containing substances. Nevertheless, this is rather ascribed to sphingosin in the molecule, because cerebron and psychosin reacted similarly. S.S. II contained cholesterol (Liebermann-Burchard) in a distinct amount and S. S. I in a minute amount, but the others did not. Exclusive of III, the substances were biuret-negative. In the quantitative respect, nitrogen varied from 3.05 to 5.07%, phosphorus from 1.07 to 3.36% and sugar as galactose by the indole reaction from 3.77 to 16.4% (The sugar of S.S.II was not determined.). In sugar analysis, the orcinol method gave lower values than the indole and diphenylamine methods, probably owing to incomplete hydrolysis.

Hemolysis by Fractions of the Tumor Lipoids.

With the aim of examining the hemolytic power, the fractions were taken up in physiological saline (The solutions and emulsions possessed pH of 5.6–6.0, excluding the emulsion (1%) of Fr. Bzhs-as whose pH lay at 4.2.) and progressively diluted with physiological saline. To 0.5 cc. of all the solutions and emulsions thus prepared 2 drops of a 3% erythrocyte suspension were added and the mixture stood in a thermostat at 37°C for 3 hours, shaking twice an hour. The sequels are seen in Table VIII. In the control, where physiological saline was used instead of a solution or an emulsion, no hemolysis took place.

Discussion.

In search for a group-specific lipoid, we have obtained four most active fractions. It will be legitimate here to assume that a single group-active compound was disseminated among the fractions, owing to the alteration of its solubility by various contaminants. If then, S.S.IV looks to be the purest fraction, for this was free from biuret-positive substances and cholesterol and, at that, contained the largest quantity, calculated from galactose, of psychosin[8] which appears to be one of the determinant groups in group lipoids (cf. Masamune[9]). This fraction was not superior to the others in the anti-agglutinative potency, but the assay employed is not so precise as to distinguish minor differences and moreover a possible co-adsorbens (cf. Hamasato[4]) might have enhanced the action of particularly S.S.I, that was poor in psychosin. That the nitrogen content of S.S.IV was more than corresponds to sphingosin (the same applies also to other S.S.'s) supports the hypothesis of Masamune[9] on the determinant structures common to all ABO group substances. Phosphate rests were also present in the group lipoids from blood cells (Hamasato[4]) and normal liver (Matuda[10]). But with these the present compound is most probably

not identical, judging from its dissimilar equivalent ratios of sugar : N : P (1 : 3 : 2 in the group lipoids from blood cells and 1 : 3 : 3 in those from liver). The accurate relationship will be disclosed by its further purification (s. Second Report).

From the findings here alone, we can not derive any conclusion concerning the grade of differentiation of group lipoids in cancer. In order to dissolve this problem, studies of the group lipoids not only from normal liver but also from gastric cancer and the mucosa of normal stomachs are now being carried on in this Institute.

The Grant Committee for Scientific Researches of the Education Department gave a grant towards the costs of this investigation, which is gratefully acknowledged. H. Masamune.

Bibliography and Notes.

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3) Fisler and Moritsch, Zeits. f. Immun. forsch., 1928, **57**, 421.
4) Hamasato, Tohoku J. Exp. Med., in press.
5) Cold dry benzene was used.
6) All the substance dissolved in benzene and was precipitated entirely by acetone.
7) Haneda, Sinokawa and Yasuoka, Tohoku J. Exp. Med., 1944, **46**, 263.
8) S. S. II was not analyzed regarding galactose, but it is assumed from its solubility to involve less of the sugar than IV.
9) Masamune, Ketsuekigaku Togikai Hokoku, I, 1948, 180; Tokyo Iji Shinshi, 1949, **66**, 269.
10) Matuda, unpublished.

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A Clinical Respiratory Recorder.

By

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There were many sorts of devices reported to record the respiratory movements of the thorax of man. Some of them, however, are too complicated for clinical purposes. A devise here reported is very simple, stable and practical (Fig. 1).

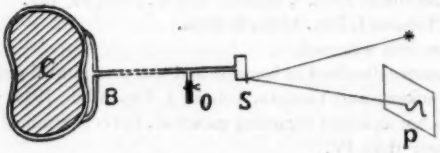

Fig. 1. Diagram of the respiratory recorder.
C: Chest. B: Rubber bag of sphygmomanometer. O: Side opening for inflation of the rubber bag. S: Segment capsule with a tiny mirror. P: Moving photographic paper.

The canvas band of the ordinary Riva Rocci's sphygmomanometer is buckled closely (but not too tightly) around the chest. The rubber bag within the band is inflated slightly through a side opening of the attached tube, which is also connected to a segment capsule. The respiratory changes in pressure inside the bag are recorded optically. The recording by means of Marey's tambour on a smoked paper will do also well. Fig. 2 is an example of the optical records.

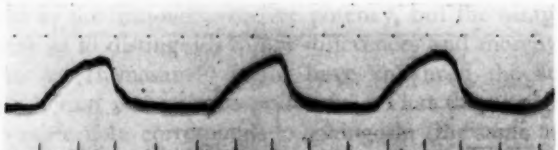

Fig. 2. Record of respiration from a man, in recumbent position. Upper: Time marking, one second. Middle: Respiration, inspiration upwards. Small notchings on the curve are due to cardiac beats. Lower: Electrocardiogram, the second lead.

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The Change in Electrical Excitability of the Human Retina caused by a Flash of Light.

By

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Introduction.

Motokawa[1] showed that the electrical excitability of the human retina, as determined with a light sensation evoked by electrical stimulation as an indicator, takes a phasic course after an illumination of the retina 1/5–2 seconds in duration, the refractory period lasting about 0.25 seconds and the supernormal phase more than several seconds. The maximum of supernormal excitability was reached in two seconds after the end of a foveal illumination with white light independent of its intensity. As no measurements were taken as to the excitability within 0.25 seconds after the end of illumination, we have tried to get a precise information about the change taking place within such a brief interval in the present investigation.

Experimental.

Apparatus and Procedure.

In order to work with an extensive range of intensity as well as duration of illumination, the following optical system was used. As shown in Fig. 1, an image of the incandescent filament of a tungsten lamp was focussed by lenses L_1 and L_2 on the plane of a revolving circular disc, 100 cm. in diameter with a variable slit. The diverging light was then paralleled by a lens L_3 and passed through the opening of a shutter, Sh, on to an opal glass, Og, which served as the illuminating field. The brightness of the illuminating field was controlled by means of three Mazda's neutral tint filters, each transmitting approximately 1/50. These filters were calibrated separately with a Lummer-Brodhun photometer. The absolute brightness of the illuminating field was measured with Mazda's Luxmeter well calibrated shortly before the experiment. The illuminating field, a circular patch 2.1 cm. in diameter, was viewed through an artificial pupil, 4 mm. in diameter and 42 cm. away from the former. When high intensities were

Fig. 1

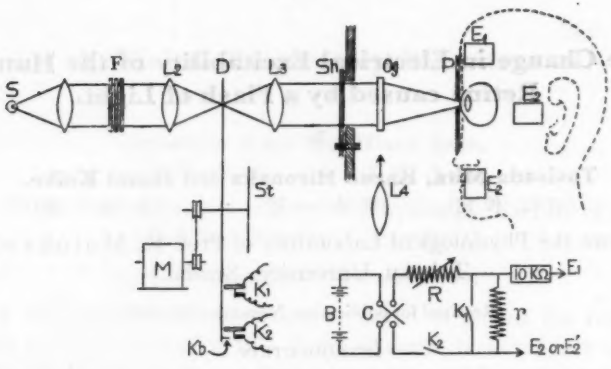

Fig. 1. Diagram of the optical and the electrical arrangements for measuring electrical excitability of the retina. B: 50 volt battery. C: Commutator. D: Revolving circular disc. E_1, E_2, E_2': Electrodes. F: Filters. K_1, K_2: Keys. Kb: Contact-breaker. L_1, L_2, L_3, L_4: Lenses. M: Electric motor. Og: Opal glass. P: Artificial pupil. R: Variable resistance. r: Fixed resistance. S: Light source. Sh: Shutter. St: Stroboscope.

necessary, the opal glass, Og, was removed and a lens L_4 was placed instead of the former. This lens gave an image of the filament at the artificial pupil and furnished about 200 times as much light as with the arrangement making use of the opal glass. In this way intensities ranging from below threshold up to 1.62×10^2 candles per square cm. were obtained. The duration was controlled by varying the width of the slit on the circular disc, which was revolving at a constant rate, i.e. one revolution every three seconds, giving repeated flashes at the same rate. The shutter, Sh, closed usually, was opened by the subject for receiving any one flash for the measurement.

Two red fixation points, brightness of which could be controlled separately by potentiometers, were placed 2 cm. apart on either side of the illuminating field.

Two silver plates of the same size, 2 cm. $\times$ 1.5 cm., were used as the electrodes for stimulation, the one, E_1, was placed at the forehead above the eye to be tested, and the other, E_2, at an intermediate region between the eye and the ear on the same side, and sometimes in the mouth (E_2') between the gums and the inner surface of the lips. As shown in Fig. 1, the stimulating voltage was regulated by a variable resistance, R, another resistance, r, being kept at a constant value 200 ohms. To reduce the effect of variation of the skin-resistance, a constant resistance of 10 kΩ was inserted in series. The following device was used for obtaining a rectangular current

pulse of difinite duration and for delivering the pulse at a given moment after the end of illumination. Two keys K_1 and K_2 arranged at a definite distance apart were opened successively with an interval of 140 msec. by a contact-breaker attached to the margin of the revolving disc. The interval between the end of illumination and the beginning of the stimulating current could be varied at will by displacing the contact-breaker, Kb, along the circumference of the disc. The direction of the stimulating current was alternated succesively, for this seemed to minimize the effect of polarization especially when the use of a relatively strong current was necessary as in our experiment.

Measurements were taken after a stay of 30 minutes in the dark, and between succesive illuminations, an interval from 30 seconds to 2 minutes according to their intensities and durations was allowed to avoid a cumulative effect of illumination. The illuminating field were fixed centrally by one eye, the other eye being covered with a black band. We used the same measure ζ as in Motokawa's experiment to represent the change of the electrical excitability. This measure is defined as follows:

$$\zeta=(E-E_o)/E_o\times 100,$$

where E_o is the electrical excitability, or reciprocal of electrical threshold of the dark-adapted eye, and E the excitability at any moment after the end of illumination.

Results.

I. General Aspect.

The results are given in Table I. As the information conveyed by the measurements can best be understood from their graphic representation, two typical sets of measurements are reproduced in Fig. 2. This figure shows that the value of ζ are negative for the most part of the first second after the end of illumination, and that they decrease at first, reach a minimum and then become positive after a certain interval.

As shown in Fig. 2B, for a given intensity, the longer the duration of illumination is, the earlier the positive value of ζ is attained. The negativity of ζ indicates a decrease of electrical excitability or a refractory phase, while the positivity means an excitability increase or a supernormal phase. The above finding shows, therefore, that the refractory period decreases with increasing duration of illumination. This fact is worthy of remark, because it seems to be against our physiological common sense.

As Fig. 2 shows, each ζ-curve reaches a minimum in about 0.05″–0.1″ after the end of illumination almost independent of its intensity and duration. The maximum of supernormality is attained in about 2 seconds after the end of illumination in all case, so long as white light is used.

TABLE I.

I \ Z / t	0.0	0.05″	0.1″	0.25″	0.5″	0.75″	1.0″	1.5″	2.0″	2.5″	T	λ
	2″	-75			-20	50	65	75		100		0.31″	$\bar{1}$.875
	1/2″	-76	-84	-76	-53	-9	32	60	80	83	83	0.56″	0.005
1.62×10^{2}	1/16″	-83	-87	-83	-63	-32	0	-30	56	65	60	0.75″	0.127
	1/250″	-87	-96.5	-91.5	-70	-55	-37	8	38	39	36	0.94″	0.393
	1/1000″	-87	-89.5	-90	-76	-61	-74	-20	13	15	14	1.04″	0.778
	2″	-82			-38	12	43	66		94	90	0.41″	$\bar{1}$.941
	1/2″	-82	-89	91	-50	-10	20	45	67	71	61	0.58″	0.107
0.808	1/16″	-82	-96	-95	-60	-30	-3	26	46	52		0.78″	0.266
	1/250″	-80	-95	-94	-60	-28	-8	12	27	30		0.90″	0.501
	1/1000″	-77	-94	-90	-55	-20	-7	3	12	15	13.5	0.97″	0.797
	2″	-60			-30	10	23	48	65	77	76	0.40″	$\bar{1}$.892
	1/2″	-66	-71	-70	-40	-12	9	40	55	62		0.59″	0.059
1.62×10^{-2}	1/16″	-76	-86	-85	-54	-29	-6	25	40	42		0.80″	0.310
	1/250″	-68	-75	-75	-42	-17	-5	12	23	24		0.90″	0.495
	1/1000″	-50	-68	-62	-27	-8	-4	5	9	10		0.92″	0.719
	30″	-44			3	15	30	48	60	66	60	0.28″	$\bar{1}$.830
	2″	-44			-14	0.5	25	38	48.5	50		0.37″	$\bar{1}$.954
	1/2″	-51.5	-53	-44	-22	5	21.5	31	37	40		0.45″	0.122
3.23×10^{-4}	1/16″	-57	-61	-56	-28	-3	12	22	26	30		0.55″	0.308
	1/250″	-45	-48	-40	-25	-6	2	12.5	15.5	18		0.68″	0.407
	1/1000″	-36	-41		-21	-8	0	5	7.5	9	9	0.75″	0.596
	30″	-14			3.5	14	27	44	50	54	50	0.20″	$\bar{1}$.413
6.46×10^{-6}	2″	-14.5			-3	10	20	26	32	37	32	0.29″	$\bar{1}$.578
	1/2″	-15	-15		-4	7	14	20	23	25		0.35″	$\bar{1}$.778
	1/16″	-17	-16		-6	0	8	12	14	17		0.50″	0.000

Change of Electrical Excitability (=ζ). I: Intensity of illumination in candles /cm². t: Durations of illumination in sec. Z: time interval between the end of illumination and electrical stimulation in sec. T: Refractory period.

$\lambda=\log_{10}(|\zeta_{mr}|/|\zeta_{ms}|)$, where ζ_{mr} and ζ_{ms} are maxima of ζ in refractory phase and in supernormal phase respectively. Foveal illumination, 1.5° in visual angle. Subject: T. Mita.

Fig. 2

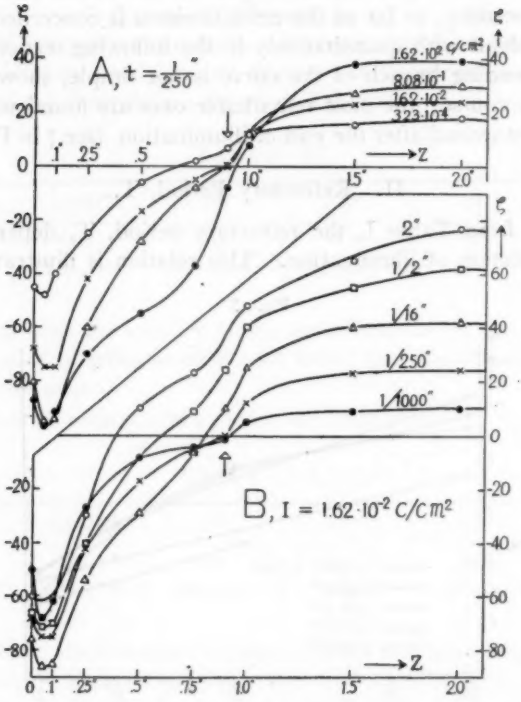

Fig. 2. Change in electrical excitability plotted as ζ against Z in sec, where Z is the time interval between the end of illumination and the beginning of electrical stimulation. A. A set of measurements for different intensities and a constant duration of illumination (1/250"). Numbers on curves indicate intensities of illumination in candles /cm². B. For different durations and a constant intensity (1.62×10^{-2} C/cm²) of illumination. Numbers on curves indicate duration of illumination in sec.

The value of ζ at the maximum increases with increasing intensities in the sets of measurements with constant duration of illumination (see Fig. 2 A) and with increasing duration when the intensity is kept constant (see Fig. 2 B).

On the contrary, the value of ζ at the minimum has no such simple relation to the intensity and duration of illumination as does the value of ζ at the maximum. The minimum stands at the lowest level for a certain intermediate duration of illumination when its intensity is kept constant, whereas in the sets of measurements with fixed duration, the lowest level of the minimum is found to be of sufficiently high intensities. In short, an

increase in duration of illumination does not act in the same sense as an increase in intensity, so far as the refractioriness is concerned. This relation will be dealt with quantitatively in the following section.

The ascending branch of the curve is not simple, showing some inflexions in its course; the most remarkable ones are found within the 2nd half of the first second after the end of illumination (see ↑ in Fig. 2 A & B).

II. Refractory Period, T.

As seen from Table I, the refractory period, T, depends strikingly upon the duration of illumination. This relation is illustrated in Fig. 3.

Fig. 3

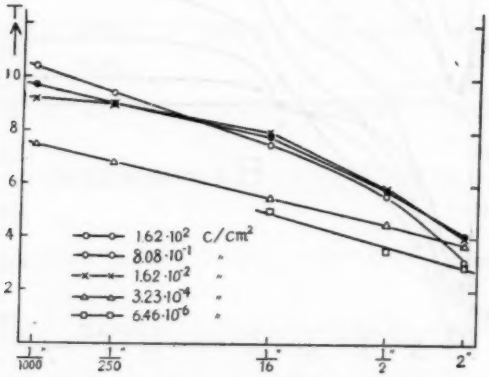

Fig. 3. The refractory period, T, in sec. is plotted against $\log_{10}t$, where t is duration of illumination in sec.

which shows that T decreases with increasing duration (t) of illumination, and that for any intensity of illumination T can be expressed approximately by the following equation,

$$T=T_o-m\log_{10}t \qquad (1),$$

where T_o and m are independent of t.

On the other hand, for any given duration of illumination, the refractory period tends to remain almost constant for a wide range of higher intensities, although it depends somewhat on intensities for a range of lower ones.

III. Effect of Intensity and Duration on Refractoriness and Supernormality.

Motokawa and Iwama[2] reported that when the duration of illumination lay between 1/5–2 seconds, the value of ζ at the maximum of super-

normality, ζ_{ms}, could be given by the following experimental formula:

$$\zeta_{ms}=a\sqrt{KI}/(1+\sqrt{KI}) \qquad (2),$$

where a and K are positive constants. We have confirmed that the formula holds approximately good for any duration of illumination. The values of constants of this formula are shown in Table II.

TABLE II.

Duration, t	1/1000″	1/250″	1/16″	1/2″	2″
a	14.8	31.3	47.4	64.1	79.6
K	7.31×10^{3}	5.21×10^{3}	4.62×10^{4}	5.93×10^{4}	9.55×10^{4}

We tested next the validity of the same formula for the value of ζ at the deepest point of refractoriness, and found that the formula held good for this case as well.

The values of constants are shown in Table III.

TABLE III.

Duration, t	1/1000″	1/250″	1/16″	1/2″	2″
a	87.6	92.8	94.7	84.7	73.0
K	2.36×10^{3}	2.94×10^{3}	7.48×10^{3}	7.19×10^{3}	8.74×10^{3}

As seen from the formula, (2), the constant a indicates the upper limit of ζ at sufficiently higher intensities, and the constant K is regarded as a proportionality coefficient dominating in sufficiently lower intensities.

Table II shows that the possible maximum of ζ in the supernormal phase decreases with decreasing duration of illumination, and that the development of supernormality is only weak for shorter durations of illumination such as 1/250″ and 1/1000″, however high the intensity may be. On the contrary, Table III shows that the constants a and K depends to a lesser degree on the duration of illumination. It follows that the possible minimum of ζ in refractoriness is approximately the same for all durations of illumination.

Thus, the effect of illumination on the maximum or minimum of ζ can be seen more clearly in supernormality than in refractoriness.

IV. Relation between Maximum Supernormality (ζ_{ms}) and Maximum Refractoriness (ζ_{mr}).

Defining by the equation as $\lambda=\log_{10}(\zeta_{mr}/\zeta_{ms})$, λ can be calculated in each series of measurements, as shown in the last column of Table I. As seen from this Table, λ for a given intensity decreases with increasing

duration. If λ is plotted against $\log_{10}t$, a linear relation is found between λ and $\log_{10}t$, in every sets of measurements with given intensity, or λ can be expressed by the following experimental formula:

$$\lambda=\lambda_0-n\ \log_{10}t \qquad (3),$$

where λ_0 and n are constants independent of t. Giving t in sec., values of λ_0 and n were obtained from the data, as shown in Table IV.

TABLE IV.

C/cm_2	$6.46.\ 10^{-6}$	$3.23.\ 10^{-4}$	$1.62.\ 10^{-2}$	0.808	$1.62.\ 10^{2}$
λ_0	−0.30	0.02	−0.02	0	−0.1
n	0.24	0.21	0.23	0.23	0.24

This table shows that λ_0 and n remains almost invariant irrespective of intensities, though λ_0 at the lowest intensity deviates somewhat from this regularity. This means that maximum refractoriness and maximum supernormality are in close relation to each other, and that their ratio depends greatly upon the duration of illumination but is almost independent of intensities except at a range of very low intensities. By eliminating $\log_{10}t$ from both equations (1) and (3), we get the relation between the reractory period (T) and λ as follows:

$$(\lambda-\lambda_0)/(T-T_0)=C \quad \text{or,} \quad \lambda=CT+C_0,$$

where C and C_o are constants.

This expression means that log (ζ_{mr}/ζ_{ms}) bears linear relation on the refractory period. In other words, the maximum supernormality, ζ_{ms}, is entirely determined by the maximum refractoriness, ζ_{mr}, and the refractory period, T.

Discussion.

In this work, we confirmed the existence of the refractory phase and the supernormal phase. We interpret this phenomenon in such a manner that even a brief illumination of a small part of the fovea causes at first inhibition and then facilitation somewhere in the visual apparatus including higher visual centers. The location of this phenomenon will, however, not be mentioned here.

We have a number of evidence showing that the refractory period lasts as long as 0.5 seconds or more, which seems to be inconsistent with Motokawa's results.[1] Our systematic study has, however, elucidated that this apparent discrepancy is due to the difference of duration of illumination in both cases; as the formula (1) shows, the refractory period decreases with increasing duration of illumination and is definitely shorter than 0.5 seconds for the illumination 2″ in duration as was the case in

Motokawa's experiment.

Motokawa[1] reported that the electrical supernormality caused by illumination of various part of retina related closely to the light-sensitivity of the respective illuminated parts; he found that the distribution of the electrical supernormality over the retina precisely coincided with that of the light-sensitivity.

Since refractoriness and supernormality represent different aspects of one and the same physiological process, the former must depend upon the location of illumination in the same manner as the latter. This view is supported by the fact that there is a quantitative relation between supernormality and refractoriness, as mentioned above.

Motokawa performed a number of investigations on the mechanism of vision by utilizing electrical supernormality as an indicator. Now we can compare the utility of refractoriness with that of supernormality for the same purpose. In general, the measurement of supernormality is much easier than that of refactoriness for the following three reasons. 1. The measurement of supernormality never calls forth such a high stimulating voltage as would cause unpleasant skin-sensation and muscular twitches. 2. A simpler apparatus will do for the measurement of supernormality, because it develops more slowly. 3. The threshold can be determined more easily and accurately, because the disturbance effected by after-images is much slighter in the supernormal phase than in the refractory phase. However, the refractoriness is superior to the supernormality in some respects. For instance, the former is a more effective indicator, for studying the effect of a very short flash, say a flash of 1/1000 seconds, because it causes a deep and long lasting refractoriness, but only a weak supernormality. In this example, the refractoriness is made deeper and longer by raising intensities, but the supernormality is scarecely increased thereby.

Motokawa[3][4] reported that maximum of electrical supernormality is displaced by altering wave-length of light in such a manner that the longer the wave-length, the shorter the interval between the end of illumination and the maximum of supernormality, for instance, the interval was 1.0″ for light from the red end of the spectrum. The use of test light sufficiently long in duration must have favoured this discovery. If he had used a very short flash, he would have never been able to discover this interesting fact, because, as shown by our experiment, only slight supernormality appears one second after the end of such a short flash. In such a case, we can take the refractoriness as the indicator for the studies of the same relation, which are now in progress in our laboratory.

Summary.

Changes in electrical excitability of the dark-adapted eye caused by a

brief illumination with white light were measured under various conditions of intensity and duration of illumination.

(1) It was confirmed that a flash of light was followed by an electrical refractory phase and an electrical supernormal phase successively, both extrema of refractoriness and of supernormality being reached in about 0.05″ and about 2″ after the end of illumination respectively.

(2) The refractory period was found to be strikingly dependent on duration of illumination but far less on its intensity.

(3) It was found that there exists a quantitative relation between refractoriness and supernormality.

(4) It was pointed out that an increase in intensity does not always act in the same sense as an increase in duration of illumination.

We wish to thank Professor K. Motokawa for his advice and helpfulness.

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Effects of Various Cations and Hydrogenions on the Action Current of Frog's Skin.

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There are many experiments on the action currents of nerve and muscle, and on the current of rest of skin, but the action current of skin has been studied not so extensively as the current of rest, probably because the experimental method hitherto employed was not suitable enough to obtain steady action currents.

Motokawa[2)3)4)] devised a convenient method for studying the action current of frog's skin and made it possible to study this phenomenon with equal accuracy as in the studies of the action current of muscle and nerve. By means of this method I have investigated the effects of various cations in various concentrations.

In this research, special attention was paid to the supernormal excitability which followed brief tetanizing stimulation of the secretory nerve of skin, for it is reported by Adrian[1)] that the supernormal phase of nerve is greatly dependent on the ionic constitution of perfusing fluid.

Method.

Experiments were performed with Hermann's nerve-skin preparation at room temperature from April to July and from September to December. The current of rest was carefully compensated, and the action current evoked by a single maximum induction shock from Porter's inductorium was measured as a deflection of a mirror galvanometer (Siemens-Halske), the period of which was 8 seconds. An interval of 3 minutes was laid between stimulations to allow complete recovery from previous excitation.

From time to time a tetanizing current lasting for 3 seconds was delivered in order to test the supernormal excitability following tetanization. As shown by Motokawa,[5)] the action current of frog's skin as tested by a single maximum induction shock is increased in magnitude for several minutes or longer after tetanization. The first test-shock was given 90 seconds after tetanization, for by this time the large deflection caused by

tetanization came to an end, and allowed an accurate measurement of the action current evoked by the test-shock. The outer surface of skin was perfused with Ringer, and solutions to be tested were applied to the inner surface. The constitution of Ringer used was as follows: NaCl 0.6%, KCl 0.02%, $CaCl_2$ 0.02%, pH of this solution 6.4. In order to obtain isotonic solutions containing cations in question in various concentrations, isotonic salt solutions of NaCl, of LiCl, of $CaCl_2$ and so on were mixed with Ringer in various proportions. The notation 2/10 NaCl will be used in the following to denote a mixture of isotonic NaCl solution with Ringer in ratio 2:8.

Results.

On stimulation of the secretory nerve of skin the action current flows in the external circuit from the outer to the inner surface of skin. This direction is expressed as "outgoing." The action current may be reversed in direction or become "ingoing" under some conditions. When the current of rest is too weak the action current is inclined to be ingoing. The following result was obtained at preparations showing always outgoing action currents. Those showing action currents of reverced direction were dis-

Fig. 1

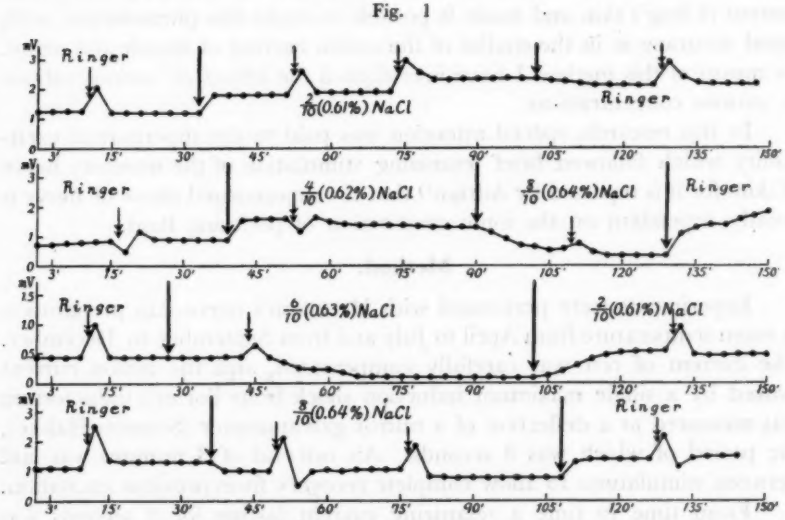


Fig. 1. Effect of NaCl on the action current of frog's skin. Ordinates: Action currents evoked by a single maximum induction shock. Abscissæ: Time in minutes. Each circle represents an action current. Double arrows indicate tetanizing stimulation of 3 seconds, and thick single arrows exchange of solutions. 2/10 NaCl denotes a mixture of isotonic NaCl solution with Ringer in ratio 2:8.

carded, because the magnitude of action currents is usually smaller and less stable in such preparations.

1. *The Effect of NaCl and LiCl.*

The result is shown in Fig. 1, in which each circle represents the magnitude in mV of the action current evoked by a single maximum induction shock. The double arrows indicate the moment at which a tetanizing stimulus was given, the deflection due to the tetanization being not shown in Fig. 1. It will be seen from the figure that the magnitude of action current 90 sec. after the tetanization is always greater than that of others. This is an expression of supernormal excitability.

The single thick arrows in the figure indicate the moment for exchange of solutions. NaCl of lower concentration than 4/10 has no remarkable effect on the action current, but the latter is reduced greatly in higher concentrations. The phenomenon is, however, satisfactorily reversible, as long as NaCl and LiCl are used.

LiCl is as little harmful as NaCl in lower concentrations, and the critical concentration for lowering action currents is about the same as with NaCl. It is to be noted that the supernormal excitability after tetanization usually disappears with decreases of action currents, and that its recovery is associated with the recovery of action currents.

2. *The Effect of KCl and NH_4Cl.*

KCl is so effective in reducing action currents that such a low concentration as 0.5/10 is sufficient for suppressing it as enormously as shown in Fig. 2. By application of 2/10 (0.3%) KCl the action current is irreversibly suppressed in 20–30 minutes.

NH_4Cl is similarly effective, but its action seems slightly less severe than KCl; the critical concentration for irreversible suppression lies between 6/10 (0.35%)–8/10 (0.47%).

3. *The Effect of $CaCl_2$, $MgCl_2$, $BaCl_2$ and $AlCl_2$.*

$CaCl_2$ in lower concentrations than 6/10 (0.5%) increases the action current, and in higher concentrations the decreasing effect is as marked as in corresponding concentrations of other salt solutions. In the former case the magnitude of action currents can be several times as great as in Ringer, but the supernormal excitability shows no corresponding increase. The reversibility is always satisfactory. However, the magnitude of action currents becomes supernormal after 6/10 (0.5%) $CaCl_2$ is removed to apply again Ringer solution. This effect is probably due to $CaCl_2$ remaining in tissue after removal of the $CaCl_2$ solution.

The action of $MgCl_2$ scarcely resembles that of $CaCl_2$ though they

Fig. 2. Effect of KCl.

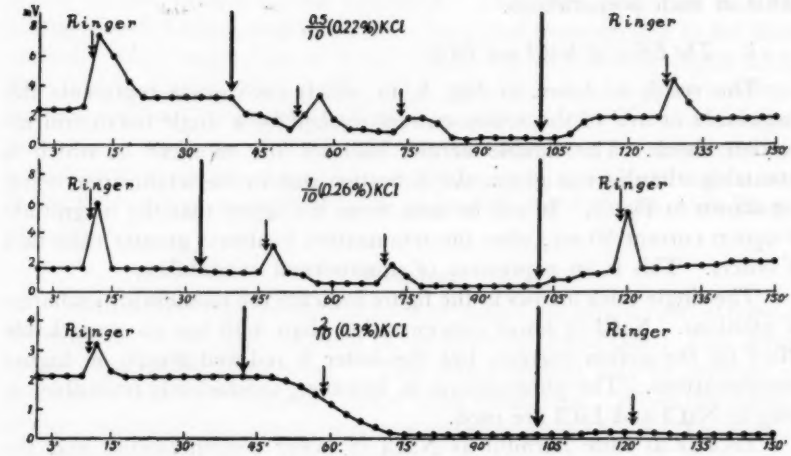
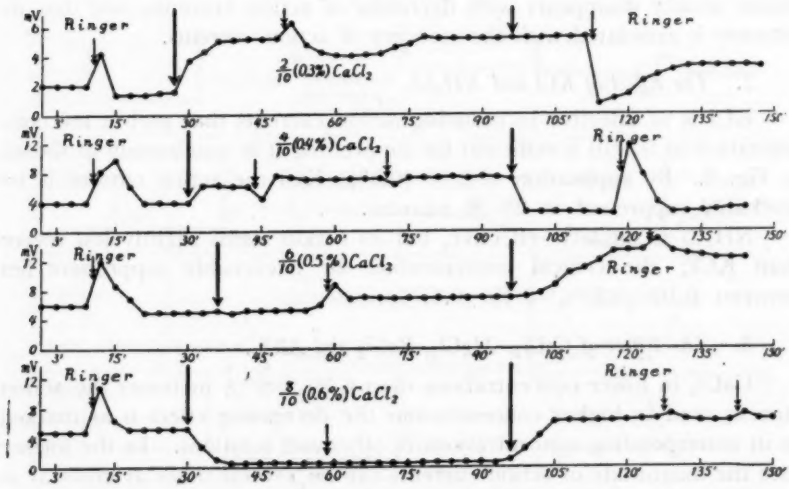


Fig. 3. Effect of $CaCl_2$.



belong to the same chemical group. $MgCl_2$ always decreases the action current in such a way that almost complete suppression ensues in higher concentrations than 4/10 (0.65%), but the reversibility is much better than in the case of KCl.

$BaCl_2$ is one of the most poisonous salts ever tested; 2/10 (0.38%)

$BaCl_2$ suppresses the action current almost irreversibly. The same is the case with $AlCl_3$.

4. *The Effect of Hydrogen ion concentration.*

Buffered Ringer solutions ranging from pH 4.4–8.2 were prepared with m/15 $NaH_2PO_4.H_2O$ and m/15 $Na_2HPO_4.12\ H_2O$ by mixing buffer solution with Ringer in ratio 1: 10, and pH of each solution was colorimetrically measured. The buffered solution was applied either to the inner or outer surface of skin.

In this experiment I found, to my surprise, that hydrogen ions in the concentration range tested have no appreciable effect whatsoever on the action current. This result corresponds with Takenaka's experiment[6] showing that periodic variations of skin-potential caused by Li-ions is almost independent of hydrogen ions in the medium.

The supernormal excitability is also little dependent on hydrogen ion concentration. This fact seems to contradict Adrian's result[1] that the supernormal excitability of nerve is determined chiefly by pH of the medium. There is, however, no evidence that the supernormal excitability of skin and that of nerve are of the same nature. The above evidence rather suggests that both phenomena are of quite different nature.

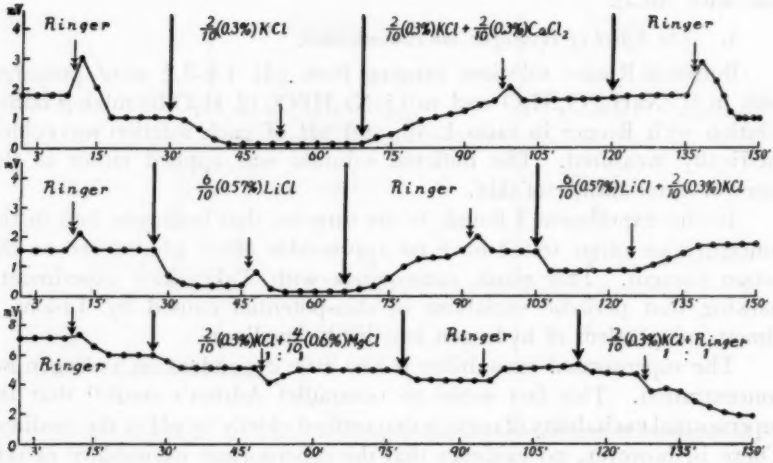
This slight effect of pH on action currents forms a striking contrast to the strong effect on the current of rest. According to Motokawa, Narita and Endo[7] the skin-potential was reduced on an average to 30% of the initial value when the pH of Ringer perfusing the outer surface of skin was changed from 7 to 5.

5. *The Antagonistic Action between Various Ions.*

As shown in section 2, KCl reduces the action current almost irreversibly in concentration 2/10 (0.3%). If the skin, the excitability of which disappeared by the action of this solution, is perfused now by a mixture of 2/10 KCl and 1/10 $CaCl_2$ in ratio 1: 1, its excitability is gradually restored, and responses to a single shock become as active as before the application of injurious 2/10 KCl. This relation is shown in Fig. 4. If the concentration of $CaCl_2$ is doubled, the action current is restored not only to the initial magnitude, but also becomes supernormal in size. Such supernormal action currents can be observed only in adequate concentration of $CaCl_2$ apart from the supernormality after tetanization.

Between KCl and $MgCl_2$ some antagonistic action is seen, though it is not so marked as that observed between KCl and $CaCl_2$. Mixing of 4/10 $MgCl_2$ with 2/10 KCl in equal amount can extinguish the harmful effect of 2/10 KCl so that the action current can remain almost normal, as shown in Fig. 4. In this case we cannot substitute Ringer for 4/10 $MgCl_2$ without decrease of action currents.

Fig. 4. Antagonistic action between ions.



Antagonistic action is seen not only between monovalent and bivalent cations, but also between two kinds of monovalent cations such as K and Li. 6/10 (0.57%) LiCl strongly reduces action currents, but if the solution is exchanged for Ringer their initial magnitude will be recovered at once. An addition of 2/10 KCl to 6/10 LiCl in ratio 1: 1 is sufficient to abolish the injurious effect of the latter, although 2/10 KCl itself is injurious enough to suppress the action current totally and irreversibly.

Discussion.

As mentioned above, the effect of cations is different according to their sort and concentration, but we can perceive some regularity. With regard to their reducing action on the action current of skin, alkali metal ions are arranged according to Hofmeister's lyotropic series:

$$K^{\cdot} > NH^{\cdot}_4 > Li^{\cdot} > Na^{\cdot},$$

and alkali earth metal ions:

$$Al^{\cdots} > Ba^{\cdot\cdot} > Mg^{\cdot\cdot} > Ca^{\cdot\cdot}.$$

Among them the action of $Ca^{\cdot\cdot}$ is worthy of note as long as it increases action currents above normal in adequate concentration, whereas all the other cations show only decreasing action. Hashida[8] and Motokawa[9] could find no such special action of $Ca^{\cdot\cdot}$ on the current of rest of frog's skin when they applied solutions to be tested to the outer surface of skin. As pointed out above, both sorts of current are differently effected by changes of pH in the medium. These discrepancies may be related to the difference in tissue involved. According to Motokawa,[10] the electrical double

layer for the current of rest is situated at the epidermic surface of skin, while the origin of the action current lies evidently in skin-glands.

Ions such as $K^{\cdot}$ and $Ca^{\cdot\cdot}$ must be highly effective on the action current because they can change the permeability of cell-membrane of glands. On the contrary, shifts of pH will scarcely cause any permeability change, at least in the skin-glands, because they normally produce acidic or alkaline secrete and are adapted to pH changes.

The fact that the order of cation-effect follows Hofmeister's lyotropic series can be interpreted from the standpoint of permeability.

The action current and the current of rest, however, are not entirely independent of each other. The direction of action currents greatly depends on the magnitude of current of rest in such a way that the action current is outgoing or ingoing according to whether the magnitude of the ingoing skin-potential is great or small. This relation will be realized if it is assumed that some part of the action current is made of depolarization current as the result of permeability increase caused by excitation. The electrical double layer which is the determining factor for the E.M.F. of the current of rest, must provide the E.M.F. for the depolarization current.

SUMMARY.

The effect of various cations on the action current of frog's skin was investigated. Solutions to be tested were applied to the inner surface of skin. The stimulus used was a single maximum induction shock.

1. The magnitude of the action current is usually decreased when Ringer is substituted for Ringer containing a great amount of alkalimetal- and alkali earth metal ions. The order of reducing effects of ions follows Hofmeister's lyotropic series:

$$K^{\cdot} > NH_4^{\cdot} > Li^{\cdot} > Na^{\cdot}.$$

The series for alkali earth metal ions is as follows:

$$Al^{\cdot\cdot\cdot} > Ba^{\cdot\cdot} > Mg^{\cdot\cdot} > Ca.^{\cdot\cdot}$$

2. $Ca^{\cdot\cdot}$ increases the action current above normal in adequate concentration.

3. Antagonistic action can be demonstrated not only between $K^{\cdot}$ and bivalent cations such as $Ca^{\cdot\cdot}$ and $Mg^{\cdot\cdot}$, but also between two kinds of monovalent cations such as $K^{\cdot}$ and $Li^{\cdot}$.

4. The action current is scarcely affected by changing pH of the medium from 4.3 to 8.2.

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Effects of Acetylcholine, Adrenalin and other Drugs on the Action Current of Frog's Skin.

By

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The innervation of skin-glands was one of the most vexed problems in physiology. Anatomists claim sympathetic innervation from morphological grounds, whereas physiologists and pharmacologists suggest parasympathetic innervation on the basis of the experimental fact that the secretion of skin-glands is stimulated by pilocarpine, and paralysed by atropine.

The discovery of acetylcholine as a chemical mediator (Loewi[1]), and Dale's classification[2] of nervous systems in adrenergic and cholinergic systems seem to have so much clarified this problem that there is no longer any obscurity about this problem. As is widely believed, the secretory nerve of skin-glands may belong to the cholinergic system, but there remains some doubt about this, because skin-glands of some animals are stimulated by adrenalin of very low concentrations.

So far as frog's skin is concerned, the effects of acetylcholine and atropine were studied in some detail (Motokawa[3] and Barnes), but no comparative study on effects of other pharmaca has been performed, though such studies are necessary to establish the physiological significance of acetylcholine as a chemical mediator at the endings of secretory nerve of the skin. Therefore, I have undertaken comparative investigations of various pharmaca in this report.

Method.

The method used was the same as described in my preceding report.[4] Pharmaca to be tested were dissolved in Ringer, and their concentration was given in per cent. The solution was applied to the inside of the skin so that it might be in contact with secretory nerve. The nerve was stimulated every 3 minutes with a single maximum induction shock, and from time to time a tetanic stimulus lasting 3 seconds was delivered in order to see supernormal excitability following tetanization discovered by Motokawa.[5] The tetanization was useful to make effects of drugs manifest

rapidly; otherwise I had to wait for long time to determine whether a drug in a certain concentration is effective at all or not.

Results.

1. *The Effect of Atropine.*

Atropine is known as a drug paralysing the secretion of various glands such as salivary, sweat-, gastric and intestinal glands. As for the skin-glands of frog, 0.01% atropine is sufficient to paralyse completely the action current in a few minutes, as shown in Table I. The supernormal action current which appears after tetanization in the normal state scarcely

TABLE I.

Effect of Atropine

Arrows indicate tetanization of secretory nerve.

Time (min.)	Action current (mV.)	Time (min.)	Action current (mV.)	Time (min.)	Action current (mV.)	Time (min.)	Action current (mV.)	Time (min.)	Action current (mV.)
	Ringer		Ringer		Ringer		Ringer		Ringer
0	10.0	0	1.9	0	2.2	0	2.5	0	0.8
3	10.0	3	1.9	3	2.2	3	2.5	3	0.8
6	10.0	6	1.9	6	2.2	6	2.5	6	0.8
9	10.0	9	1.9	9	2.2	9	2.5	9	0.8
12	→40.0	12	→3.9	12	→3.1	12	→2.6	12	→0.2
15	12.0	15	1.9	15	3.0	15	2.5	15	0.4
18	10.0	18	1.8	18	3.0	18	2.5	18	0.6
21	10.0	21	1.8	21	3.0	21	2.5	21	0.8
24	10.0	24	1.8	24	3.0	24	2.5	24	0.8
	0.1% Atropine		0.01% Atropine	27	3.0 0.001% Atropine		0.0001% Atropine	27	0.8 0.00001% Atropine
27	0.1	27	0.1			27	2.2		
30	0.1	30	0	30	2.1	30	2.0	30	0.8
33	0.1	33	0	33	1.9	33	2.0	33	0.8
36	0.1	36	0.1	36	1.8	36	→1.1	36	0.8
39	→0.1	39	→0.1	39	1.5	39	1.0	39	0.8
42	0.1	42	0.05	42	→0.4	42	1.0	42	→0.2
45	0.1	45	0	45	2.0	45	1.0	45	0.4
48	0.1	48	0	48	1.7	48	1.0	48	0.6
51	0.05	51	0	51	1.6	51	1.0	51	0.8
54	0.05	54	0.01	54	1.5	54	1.0	54	0.8
	Ringer	57	0.01	57	0.9		Ringer	57	0.8
57	0.05	60	→0	60	0.9	57	1.2	60	0.8
60	0	63	0		Ringer	60	1.5		Ringer
63	0	66	0	63	2.0	63	2.1	63	0.8
66	0		Ringer	66	2.4	66	2.1	66	0.8
69	→0	69	0	69	2.4	69	→1.0	69	0.8
72	0	72	0	72	2.4	72	1.5	72	0.8
75	0	75	0	75	→3.2	75	2.0	75	→0.2
78	0	78	0	78	2.5	78	2.3	78	0.4
81	0	81	→0.01	81	2.5			81	0.5
		84	0	84	2.5			84	0.6
		87	0					87	0.8
		90	0					90	0.8
		93	0					93	0.8
		96	0						

appears under atropine action. The critical concentration for lowering action currents is about 0.0001–0.00001%. At such a low concentration as is apparently ineffective the effect can become manifest on tetanization, and this is difficult to interpret as the result of summation of fatigue due to tetanization and of atropine effect, because recovery of action currents from their depressed state is facilitated similarly by tetanization. A plausible explanation will be as follows: The excitable tissue becomes more accessible to atropine as the result of permeability increase caused by tetanization, so that atropine effect becomes manifest. In recovery experiments the same process facilitates diffusion of atropine out of the tissue to effect more rapid recovery than without tetanization.

It is to be noted that the reversibility of atropine effect is relatively

TABLE II.

Effect of Adrenalin.

Time (min.)	Action current (mV.)	Time (min.)	Action current (mV.)	Time (min.)	Action current (mV.)	Time (min.)	Action current (mV.)	Time (min.)	Action current (mV.)
	Ringer		Ringer		Ringer		Ringer		Ringer
0	1.5	0	3.1	0	0.8	0	5.1	0	3.0
3	1.5	3	3.1	3	0.8	3	5.1	3	3.0
6	1.5	6	3.1	6	0.8	6	5.1	6	3.0
9	1.5	9	3.1	9	0.8	9	5.1	9	3.0
12	→4.0	12	→3.5	12	→3.5	12	→7.0	12	→6.3
15	2.8	15	4.5	15	0.5	15	5.1	15	4.0
18	1.0	18	3.5	18	0.5	18	5.1	18	2.6
21	1.0	21	3.1	21	0.5	21	5.1	21	2.6
24	1.0	24	3.1	24	0.5	24	5.1	24	2.6
27	1.0	27	3.1	27	0.5	27	5.1	27	2.6
30	1.0	30	3.1	30	0.5		0.0001%		0.00005%
	0.05%		0.001%		0.0005%		Adrenalin		Adrenalin
	Adrenalin		Adrenalin		Adrenalin	30	1.2	30	2.6
33	0	33	0.5	33	0	33	1.2	33	2.6
36	0	36	0.5	36	0	36	1.2	36	2.6
39	0	39	0.5	39	0	39	→3.8	39	2.6
42	0	42	0.5	42	0	42	5.8	42	2.6
45	0	45	0.5	45	0	45	4.0	45	→6.0
48	→0	48	→0.5	48	0	48	4.0	48	3.0
51	0	51	0.5	51	→0	51	4.0	51	2.0
54	0	54	0.5	54	0	54	4.0	54	2.0
57	0	57	0.5	57	0	57	4.0	57	2.0
60	0	60	0.5	60	0	60	4.0	60	2.0
63	0	63	0.5	63	0	63	4.0	63	2.0
66	0		Ringer	66	0		Ringer	66	2.0
	Ringer	66	0.5			66	4.0	69	Ringer
69	0	69	0.5			69	4.0	69	1.8
72	0	72	0.5			72	4.0	72	1.8
75	0	75	0.5			75	4.0	75	1.8
78	0	78	→0.5			78	→5.0	78	→5.0
81	→0	81	0.5			81	6.1	81	2.4
84	0	84	0.5			84	7.0	84	1.8
87	0	87	0.5			87	7.0	87	1.8
90	0					90	5.9	90	1.8
						93	5.9	93	1.8

good in comparison with other similarly effective drugs.

2. *The Effect of Adrenalin.*

Adrenalin is so effective in reducing action currents that the critical concentration is as low as 0.0001%. A characteristic feature of adrenalin is that it acts promptly and reaches its maximum effect immediately after application. It has something of all or nothing character in its mode of action. In such a low concentration as a little above the critical concentration the effect is seen only at the beginning of application, the action current soon recovering to its initial level in Ringer. This is probably due to inactivation of adrenalin by some process such as oxidation. When an adrenalin solution is effective at all, depressed action currents show no tendency to recovery in Ringer, and we see also here its all or nothing character.

TABLE III.

Effect of Acetylcholine.

Time (min.)	Action current (mV.)	Time (min.)	Action current (mV.)	Time (min.)	Action current (mV.)	Time (min.)	Action current (mV.)	Time (min.)	Action current (mV.)	Time (min.)	Action current (mV.)
	Ringer		Ringer		Ringer		Ringer		Ringer		Ringer
0	1.0	0	2.4	0	5.0	0	1.0	0	1.2	0	1.2
3	1.0	3	2.4	3	5.0	3	1.0	3	1.2	3	1.2
6	1.0	6	2.4	6	5.0	6	1.0	6	1.2	6	1.2
9	1.0	9	2.4	9	5.2	9	1.0	9	1.2	9	1.2
12	→4.0	12	→12.0	12	→3.8	12	→3.2	12	→2.2	12	→1.1
15	2.2	15	1.2	15	2.9	15	1.2	15	1.2	15	1.0
18	2.0	18	1.2	18	3.8	18	1.0	18	1.2	18	0.9
21	2.0	21	1.2	21	4.0	21	1.0	21	1.2	21	0.5
24	2.0	24	1.2	24	4.0	24	1.0	24	1.2	24	0.5
27	2.0	27	1.2	27	4.0	27	1.0	27	1.2	27	0.5
	0.1% Acetyl-choline		0.05% Acetyl-choline		0.01% Acetyl-choline		0.001% Acetyl-choline		0.0001% Acetyl-choline		0.00001% Acetyl-choline
30	2.8	30	3.0	30	11.0	30	1.5	30	1.2	30	1.0
33	2.8	33	4.8	33	6.0	33	2.4	33	1.2	33	0.5
36	2.9	36	4.0	36	6.5	36	2.9	36	1.2	36	0.5
39	2.9	39	4.0	39	7.0	39	3.1	39	1.2	39	0.5
42	2.9	42	4.0	42	8.0	42	3.5	42	→2.0	42	0.5
45	2.9	45	→4.0	45	9.0	45	4.0	45	1.8	45	→1.0
48	→5.0	48	4.0	48	→12.0	48	4.2	48	1.5	48	0.5
51	2.9	51	4.0	51	8.0	51	4.5	51	1.2	51	0.5
54	2.9	54	4.0	54	9.0	54	→1.8	54	1.2	54	0.5
57	2.9	57	4.0	57	9.0	57	1.8	57	1.2	57	0.5
60	2.9	60	→4.0	60	→6.0	60	2.2	60	→2.0		
63	→3.5	63	4.0	63	7.0	63	3.0	63	1.8		
66	2.5	66	4.0	66	7.0	66	3.0	66	1.5		
69	2.0			69	7.0	69	3.0	69	1.0		
72	1.8			72	7.0			72	1.0		
75	1.5							75	1.0		
78	1.3										
81	1.0										
84	0.5										
87	0.4										

3. *The Effect of Acetylcholine.*

According to Motokawa and Barnes, application of acetylcholine causes changes of skin-potentials similar to action currents caused by tetanization of nerve. In my experiment, too, such changes were occasionally observed at the very beginning of application of acetylcholine solution, but I did not further studied this phenomenon, and directly my attention to the effect on the magnitude of action currents.

Action currents are increased above normal by acetylcholine unless its concentration is too high. The supernormal excitability after tetanization can be seen in some cases, but the magnitude of single action currents decreases temporarily after tetanization in most cases, and increases again to the level before tetanization or over it.

4. *The Effect of Pilocarpine.*

The effect of pilocarpine is similar to that of acetylcholine at least in

TABLE IV.

Effect of Pilocarpine

Time (min.)	Action current (mV.)	Time (min.)	Action current (mV.)	Time (min.)	Action current (mV.)	Time (min.)	Action current (mV.)	Time (min.)	Action current (mV.)
	Ringer		Ringer		Ringer		Ringer		Ringer
0	5.0	0	3.2	0	1.1	0	6.0	0	2.0
3	5.0	3	3.2	3	1.1	3	6.0	3	2.0
6	5.0	6	3.2	6	1.1	6	6.0	6	2.0
9	5.0	9	3.2	9	1.1	9	6.0	9	2.0
12	→7.3	12	→3.6	12	→3.0	12	→7.0	12	→0.9
15	7.3	15	3.0	15	1.1	15	5.2	15	1.0
18	7.3	18	3.0	18	0.5	18	5.0	18	1.2
21	7.3	21	3.0	21	0.5	21	3.8	21	1.5
24	7.3	24	3.0	24	0.5	24	2.8	24	1.8
	0.05% Pilocar-pine		0.01% Pilocar-pine		0.005% Pilocar-pine	27	2.8	27	2.0
						30	2.8	30	2.0
							0.001% Pilocar-pine		0.0001% Pilocarp-ine
27	4.8	27	2.8	27	3.0				
30	4.8	30	2.8	30	3.0				
33	4.2	33	2.8	33	3.0	33	3.0	33	1.8
36	4.1	36	→2.4	36	→5.0	36	3.0	36	1.8
39	→5.2	39	2.4	39	2.4	39	2.0	39	1.8
42	4.1	42	2.4	42	1.8	42	5.5	42	1.8
45	3.0	45	2.4	45	1.2	45	5.5	45	→1.2
48	3.0	48	2.4	48	1.0	48	5.5	48	1.2
51	3.0	51	2.4	51	1.0	51	5.5	51	1.5
54	3.0			54	1.0	54	→7.0	54	2.0
57	3.0			57	1.0	57	7.0	57	2.1
60	3.0					60	6.0	60	2.3
						63	5.0	63	2.5
						66	4.0	66	2.5
						69	3.0	69	2.5
						72	3.0	72	2.5
						75	3.0		
						78	3.0		

such points as they increase action currents in middle and lower concentrations, and decrease them in higher concentrations. The critical concentration is about 0.001% for the increasing effect, and that for the decreasing effect about 0.01%. So the range of concentration for augmenting action is much narrower than that of acetylcholine.

5. *Mutual action of Atropine, Adrenalin, Acetylcholine, Eserine and Pilocarpine.*

As shown in Fig. 1, Action currents abolished by the action of 0.01% atropine is not restored by 0.05% acetylcholine which greatly increases

Fig. 1

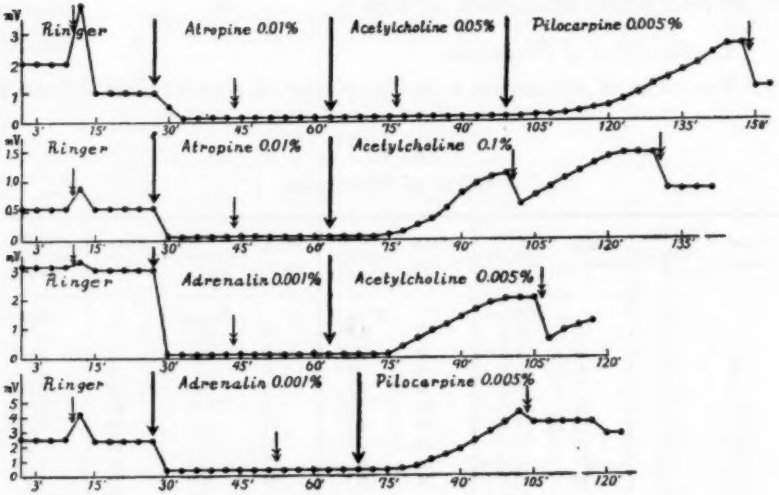

Fig. 1. Effects of atropine and adrenalin and their antagonistic action against acetylcholine and pilocarpine.

Ordinates: action currents in mV. Each circle shows the magnitude of a single action current. Abscissæ: time in minutes. Double arrows indicate tetanization of nerve, and thick arrows change of solutions.

normal action currents in Ringer. However, 0.005% pilocarpine is effective for restoration of so decreased action currents. It is interesting that 0.1% acetylcholine which depresses normal action currents, is capable of restoring those abolished by 0.01% atropine not only to the normal level, but also far above it.

0.005% acetylcholine has no recovering effect at all at atropinized preparations, but is able to restore action currents suppressed by the action of 0.001% adrenalin. 0.005% pilocarpine acts in the same way as acetyl-

choline on the action currents depressed by adrenalin.

Eserine is said to have a facilitating action on the effect of acetylcholine by suppressing the effect of cholinesterase. In my experiment, however, almost no effect could be observed when eserine was used in concentration 0.005–0.01% either singly or in combination with 0.001–0.05% acetylcholine.

Experiments with vitamin B_1 ended also in negative results.

Discussion.

The fact that action currents of frog's skin are decreased by atropine and increased by acetylcholine surely gives support to the view that the secretory nerve of frog's skin belongs to the cholinergic system. Adrenalin is equally effective, but mode of action is quite different from that of acetylcholine; the latter increases action currents in middle and lower concentrations, whereas adrenalin always decreases them. This behavior makes it unlikely that adrenalin is a chemical mediator at the endings of the secretory nerve.

0.05% acetylcholine which is effective in augmenting action currents in the normal state, is quite ineffective on atropinized preparations. Althogh antagonism between atropine and acetylcholine is so marked, the former cannot inhibit the action of pilocarpine, which increases action currents in fairly low concentrations such as 0.005% at preparations previously paralysed by 0.01% atropine. No such marked antagonism can be observed between adrenalin and pilocarpine as between atropine and pilocarpine; action currents depressed by 0.001% adrenalin increase gradually under the action of 0.005% pilocarpine, and reach the normal level in Ringer. Adrenalin cannot inhibit also the action of acetylcholine. This evidence shows that the depressing effect of adrenalin is based on some mechanism quite different from that involved in atropine action. According to Loewi and others, atropine paralyses cholinergic nerve such as vagus indirectly by inactivating acetylcholine set free at nerve endings on nerve stimulation. On the contrary, as for the effect of adrenalin some direct action must be considered. It is, however, difficult to say whether adrenalin plays some important rôle in secretion of the skin-glands of frog.

Summary.

Effects of various pharmaca such as atropine, adrenalin, acetylcholine etc. on action currents of frog's skin were investigated in order to contribute something to the problem of chemical transmission at endings of secretory nerve of the skin.

1. Atropine either abolishes or depresses action currents in higher

concentrations than 0.0001%.

2. Adrenalin acts in the same way, and the critical concentration is about 0.0001%.

3. Acetylcholine depresses action currents in higher concentrations such as above 0.1%, but increases them above normal in middle and lower concentrations.

4. Pilocarpine acts in the same way as acetylcholine. The only difference consists in that its action is not inhibited by 0.01% atropine, while acetylcholine is entirely ineffective at preparations paralysed by 0.01% atropine.

5. Though adrenalin is able to abolish action currents, it is unable to inhibit the increasing action of acetylcholine and pilocarpine.

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